New Ways in Forest Genetics

Voies nouvelles en génétique forestière

CANADIAN
TREE IMPROVEMENT
ASSOCIATION

PROCEEDINGS

TWENTIETH MEETING
PART 2

QUEBEC CITY, QUEBEC
AUGUST 19 - 22, 1985

ASSOCIATION
CANADIENNE
POUR L'AMÉLIORATION
DES ARBRES

COMPTES RENDUS

VINGTIÈME CONFÉRENCE
2^e PARTIE

QUÉBEC (QUÉBEC)
DU 19 AU 22 AOÛT 1985

EDITORS RÉDACTEURS
F. CARON
A.G. CORRIVEAU
T.J.B. BOYLE

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OF THE

CANADIAN TREE IMPROVEMENT

ASSOCIATION

PART 2:

SYMPOSIUM ON

NEW WAYS IN FOREST GENETICS

HELD IN
QUEBEC CITY, QUEBEC
AUGUST 19 - 22, 1985

EDITORS: F. CARON, A.G. CORRIVEAU & T.J.B. BOYLE

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DE LA

VINGTIÈME CONFÉRENCE

DE

L'ASSOCIATION CANADIENNE POUR

L'AMÉLIORATION DES ARBRES

2e PARTIE:

COLLOQUE SUR

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TENUE À
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DU 19 AU 22 AOÛT 1985

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Ottawa, 1986

PROCEEDINGS OF THE TWENTIETH MEETING OF THE CANADIAN TREE IMPROVEMENT ASSOCIATION

With the compliments of the Association

Enquiries may be addressed to the authors or to Mr. J.F. Coles, Executive Secretary, C.T.I.A./A.C.A.A., c/o Ontario Tree Improvement Council, Johnston Hall, University of Guelph, Guelph, Ont. N1G 2W1.

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The Twenty-first Meeting of the Association will be held in Truro, Nova Scotia, August 17-21, 1987. Speakers will be invited to address the topic of "Progressing together on Co-operative Tree Improvement Programs in Canada". Canadian and foreign visitors are welcome. Further information will be distributed in the winter 1986 to all members and to others on request. Enquiries concerning the 21st Meeting should be addressed to: Mr. Howard Frame, Tree Breeding Centre, Dept. of Lands and Forests, Debert, N.S. BOM 1GO.

To:

T.J.B. Boyle, Editor, C.T.I.A./A.C.A.A.
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Les demandes de renseignements peuvent etre adressées aux auteurs ou à J.F. Coles, Secrétaire général, A.C.A.A./C.T.I.A., c/o Ontario Tree Improvement Council, Johnston Hall, University of Guelph, Guelph, Ont. N1G 2W1.

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La vingt et unième conférence de l'association aura lieu à Truro, en Nouvelle-Écosse, du 17 au 21 août 1987. Des orateurs seront invités à adresser le sujet de "Nos efforts vis-à-vis les programmes coopératifs de l'amélioration des arbres au Canada". Les intéressés au Canada et à l'étranger sont les bienvenus. Des renseignements supplémentaires seront distribués au cours de l'hiver de 1986 à tous les membres et à tous ceux qui en feront la demande. Si vous avez des questions à poser concernant la 21^{e} conférence veuillez les adresser à: M. Howard Frame, Tree Breeding Centre, Dept. of Lands and Forests, Debert, N.S. B0M 1G0.

À:
T.J.B. Boyle, rédacteur, A.C.A.A./C.T.I.A.
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On behalf of the Canadian Tree Improvement Association, I gratefully acknowledge the Canadian Forestry Service, the ministère de l'Énergie et des Ressources du Québec, the Faculty of Forestry and Land Surveying of Laval University and the forest industries Abitibi-Price, James MacLaren, Domtar, Irving Pulp and Paper, Proctor and Gamble Cellulose, Reed Paper and Consolidated-Bathurst for their support towards the icebreakers, simultaneous translation, banquet, and field trips of our Twentieth Biennial Meeting.

We are grateful to Gilles Vallée (symposium), Gaétan Daoust and Jean Beaulieu (local arrangements), and support staff Franco Gabbino, Roger Keable, Jean-Claude Boutin, Michelle Poulin and Joyce Gardiner for their inestimable assistance.

The Association is also indebted to Jean Turgeon and Peter De Groot of the Forest Pest Management Institute, to Ben Wang and Yves Lamontagne of the Tree Seed Working Group, and to Clayton Keith of Forintek Canada Corp. and Jean Poliquin of Laval University for their organization of the workshops on Seed and Cone Pest and Wood quality, respectively.

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Au nom de l'Association Canadienne pour l'Amélioration des Arbres, je tiens à exprimer mes sincères remerciements au Service Canadien des Forêts, au ministère de l'Énergie et des Ressources du Québec, à la Faculté de foresterie et de géodésie de l'Université Laval ainsi qu'aux industries forestières Abitibi-Price, James MacLaren, Domtar, Irving Pulp and Paper, Proctor and Gamble Cellulose, Les Papiers Reed et Consolidated-Bathurst pour leur aide financière aux cocktails de bienvenue, à l'interprétation simultanée des conférences, au banquet de clôture et aux visites sur le terrain effectuées pendant notre vingtième congrès biennal.

Nous témoignons notre reconnaissance à Gilles Vallée (symposium), Gaétan Daoust et Jean Beaulieu (organisation locale), ainsi qu'à Franco Gabbino, Roger Keable, Jean-Claude Boutin, Michelle Poulin et Joyce Gardiner pour leur aide inestimable.

Notre association est également redevable à Jean Turgeon et Peter De Groot de l'Institut pour la répression des ravageurs forestiers, à Ben Wang et Yves Lamontagne du Groupe de travail sur les semences forestières ainsi qu'à Clayton Keith de Forintek Canada Corp. et Jean Poliquin de l'Université Laval pour l'organisation des ateliers sur les ravageurs des semences et des cônes et sur la qualité du bois, respectivement.

Armand Corriveau
Président

NOTE D'OUVERTURE/INTRODUCTORY REMARKS

Good morning, Ladies and Gentlemen:

Welcome to Québec. Welcome to the 20th meeting of the Canadian Tree Improvement Association. My name is Armand Corriveau, I am the chairman of the Organizing committee. As you may note, this room is provided with simultaneous translation facilities. Please do not hesitate to use the headsets at your disposition if needed. Our invited lecturers will speak either in English or French.

Bonjour, Mesdames et Messieurs,

Bienvenue au 20^{e} congrès de l'Association Canadienne pour l'Amélioration des Arbres. Mon nom est Armand Corriveau, je suis le président du comité d'organisation. Comme vous êtes à même de le constater, cette salle est pourvue des services de traduction simultanée. Nous vous prions donc d'utiliser les écouteurs mis à votre disposition s'il s'avérait que ce soit nécessaire, puisque nos conférenciers invités s'adresseront à vous soit en français ou en anglais.

Le défi que nous avons à relever, nous, biologistes de la forêt, généticiens et améliorateurs des arbres, est de taille.

En effet, les organismes avec lesquels nous travaillons possèdent toutes les caractéristiques propres à rendre difficile l'acquisition des connaissances et des gains génétiques et économiques que nous recherchons:

- ils sont de longue vie et de fortes dimensions;
- leur reproduction est tardive et irrégulière;
- de plus ils sont généralement difficiles à multiplier par voie végétative.

Nous demeurons cependant optimistes puisque de nouvelles et prometteuses avenues s'ouvrent à nous:

"Voies nouvelles en génétique forestière - New ways in forest genetics": tel est le thème de notre congrès.

Ces nouvelles biotechnologies, approches et sphères de recherche que sont l'induction florale, les analyses enzymatiques, la sélection et l'utilisation d'organismes symbiotiques, la micropropagation et la culture de tissus, de même que la fusion de protoplasme et le génie génétique nous permettent d'espérer des progrès plus rapides en génétique forestière et en amélioration génétique des arbres.

Ces voies nouvelles nous seront exposées de façon éloquente par nos conférenciers invités. Lors des présentations volontaires aussi bien que lors des séances d'affichage et des visites en laboratoire, nous serons à même de constater tout le dynamisme qui caractérise et toutes les possibilités qu'offre la recherche dans ces domaines.

En terminant, je vous souhaite un très fructueux congrès et un agréable séjour en notre ville et j'invite le nouveau Directeur général régional du Service canadien des forêts à Québec, le Dr Yvan Hardy, et M. Jean-Claude Mercier, sous-ministre associé (Forêts), Service canadien des forêts, à vous adresser la parole.

Armand Corriveau
Président

HOMMAGE À MARK JOHANNES HOLST

Nous avons raison d'être optimistes lorsque nous regardons en arrière et constatons les progrès significatifs que nous avons réalisés ainsi que le travail abattu par nos prédécesseurs. Ces pionniers dépourvus de grands moyens, compensaient par la détermination, l'amour de leur science et du travail bien fait.

Il y a 35 ans cette année, Mark Johannes Holst entreprenait ses travaux d'amélioration génétique des arbres à la Station forestière expérimentale de Petawawa. Mark est décédé en novembre 1983 des suites d'une longue maladie héréditaire.

C'est en hommage à sa mémoire et à son oeuvre que nous dédions ce vingtième congrès de l'Association Canadienne pour l'amélioration des arbres. Il fut l'un de ses membres les plus dévoués.

Pour nous remémorer la personnalité et l'oeuvre de ce pionnier canadien de l'amélioration des arbres, nous accueillons le Dr Kris Morgenstern, ancien collègue et ami de Mark.

HOMAGE TO MARK HOLST

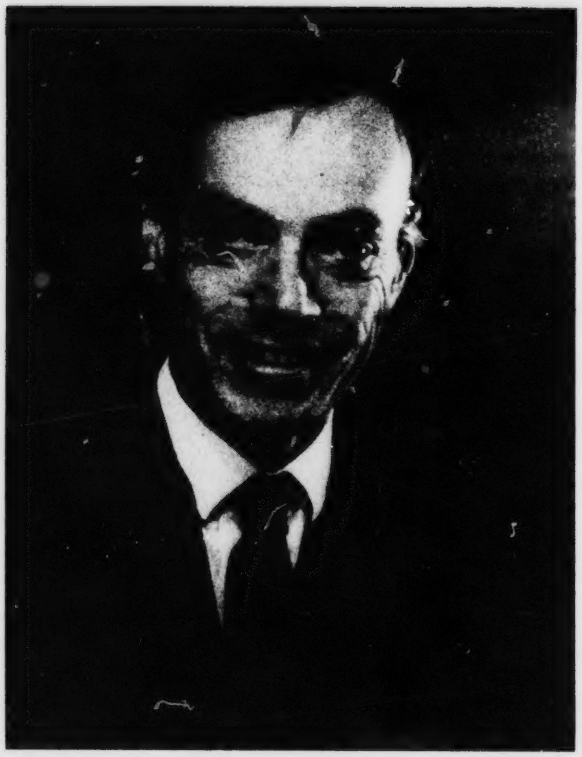
We have to be optimistic when we look back and acknowledge the significant progress that we have made and the many achievements accomplished by our predecessors. These pioneers compensated the lack of adequate resource by their unyielding determination and love of science and good work.

In 1950, 35 years ago this year, Mark Johannes Holst undertook tree breeding research at Petawawa Forest Experiment Station. He retired in 1976 and died in November 1983 after a long illness.

We dedicate this twentieth meeting of the Canadian Tree Improvement Association to his memory and to his work. He had been one of the most devoted members of the Association.

Kris Morgenstern, former colleague and close friend of this Canadian pioneer of tree improvement, well recall for us his personality and professional achievement.

Armand Corriveau



Mark Holst

MARK HOLST — PIONEER TREE BREEDER

E.K. Morgenstern
Faculty of Forestry, University of New Brunswick
Fredericton, N.B. E3B 6C2

Mark Holst was an active member of our Association for many years. He had a very productive career and gave strong impulses to tree breeding in Canada. It is an honour to review some of his accomplishments today.

Mark Johannes Holst was born on 27 March 1922 in Copenhagen, Denmark, and died on 14 November 1983 at Ottawa, Canada. He graduated in 1947 from the Royal Veterinary and Agricultural College in Copenhagen and retired due to ill health from the Petawawa Forest Experiment Station in 1976. For 30 years he actively pursued his interests in forest genetics and tree breeding, working in Denmark, Sweden, and Canada.

When Mark came to Canada in 1950, conditions were very different from those we find today. Reforestation was practiced on a small scale and research was very limited, but Petawawa was a good place to start: the Station contained some interesting plantations of native species and exotics, and material from interspecific hybridization, vegetative propagation and selection initiated by Carl Heimburger, John Farrar, and L.V.P. Johnson. Mark possessed a great deal of imagination, energy and enthusiasm and was well trained. Following his education in Denmark he had spent some time in Sweden. He was familiar with classical European silviculture and modern theories on tree breeding: the ideas of his teacher Syrach Larsen on selection, vegetative propagation and seed orchards, and also Olof Langlet's provenance research, and Bertil Lindquist's and Helge Johnsson's theories on selection. Due to continuous progress during the 1940's when other nations were at war, Sweden had become the leader in forest genetics. Thus when Mark arrived at Petawawa in 1950, he came with a wealth of knowledge and experience.

Mark's approach to tree breeding in Canada was characterized by openess and flexibility. Rather than repeating what was done in Denmark, he devised strategies more appropriate for Canadian conditions. The most common realization of this approach was not a small local trial for subjective observation, but a randomized and replicated experiment capable of objective quantitative analysis. He distributed such experiments to many test locations and often made them available to cooperators in other countries. Experiments of this kind were a novelty in Canada at that time.

Another aspect of this attitude was to be open to opportunities provided by nature. He knew that certain traits could best be measured in unusual or extreme years. Therefore, in these years, he mustered all resources to score resistance to frost, insects or diseases. Or opportunities arose in some years to use open pollination to good advantage. For example, in 1965, trees in a 12-year-old white spruce nursery experiment flowered abundantly. By removing the male flowers from these small trees and allowing open pollination by local white spruce, provenance hybrids were generated at low cost.

There is a third aspect of Mark's personality that was appealing. He had a good sense of humour, was an optimist, and he communicated well. He traveled widely and learned a lot - two important things for a newcomer. This had many useful consequences. He soon met many of Canada's leading foresters and made contacts. During the progressive and expansionary period of the 1950's, his ideas were well received. Under the auspices of the Woodlands Section of the Canadian Pulp and Paper Association, meetings were held to organize cooperation with companies, provincial forest services, and universities. This accelerated the establishment of provenance experiments, for example, for white spruce and jack pine. Many of these organizations participated in seed collection, production of stock, and preparation of test sites. Within Ontario, he developed excellent relationships with Carl Heimburger at Maple which helped greatly in program planning and cooperation. Furthermore, when the Committee on Forest Tree Breeding in Canada was organized in 1953, Mark became one of its most active members.

While the work in Canada grew by leaps and bounds, he still maintained many international contacts. This facilitated the acquisition of seed and plant material and also brought a constant stream of visitors and new ideas from abroad. Visitors included such well known scientists as Risto Sarvas and Max Hagman from Finland, Bertil Lindquist and Milan Simak from Sweden, Hans Schönbach and Wolfgang Langner from Germany, Dennis Richardson and John Matthews from the United Kingdom, Mirko Vidakovic from Yugoslavia, S.K. Hyun from Korea, and Hans Nienstaedt and Jonathan Wright from the U.S.A.

Several staff members contributed substantially to the progress of the work at Petawawa. Three highly competent and dedicated people were involved with the program during Mark's time for about 20 years or more: John Santon, who was responsible for records, the nursery and plant propagation; Paul Viidik, field plantations; and C.W. (Kit) Yeatman, geneticist. Each of them brought his own special skills to the program and contributed to its success.

There are so many aspects to Mark's work at Petawawa that it is difficult to describe it adequately. Not the least is the opportunity it created for others: the genetics programs in other regions of Canada which it stimulated and supported, and the young scientists who made a start under his guidance. He headed a good team, but his ideas were dominant during the first 10-15 years. Here are some of the major accomplishments of the program during his time:

1. The development of grafting and pollination techniques for the major conifer species in continental eastern Canada.
2. Design and application of a record or pedigree system for all plant material grown and distributed from Petawawa.
3. Development of provenance test series in each of red pine, jack pine, white spruce, European and hybrid larch, Scots pine and Norway spruce. Results on genetic variation patterns including disease resistance have often been surprising. They have been applied in numerous silvicultural and breeding programs, and in the development of seed zones from Coast to Coast.

4. Establishment of numerous genetic or physiological experiments with a variety of objectives including stimulation of flowering, intra- and interspecific hybridization, and development of genetic parameters. Many of the results obtained are now used in breeding programs.
5. Establishments of gene banks for many native and introduced species in arboreta and plantations. For example, the Scots Pine Christmas tree clones assembled by Mark are now used by the Ontario Christmas Tree Growers Association. The total area of genetic plantations and arboreta is about 200 ha at Petawawa alone.

Finally, Mark is the author or co-author of 30 scientific and technical publications. He also wrote 17 progress reports for the Proceedings of the Committee on Forest Tree Breeding in Canada and the Canadian Tree Improvement Association, and 20 internal reports. Many of his papers were marked by broad biological insights and they often contained good practical ideas. He certainly did not subscribe to the idea of narrow specialization.

In meeting the challenges of Canadian forestry and tree breeding during the quarter century of his work here, Mark Holst opened a path for others to follow. He was truly a pioneer, and his experiments constitute a treasure from which science will benefit for years to come.

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Conférenciers invités/Guest speakers.
De gauche à droite/From left to right: Dr. Francis C. Yeh, Daniel Cornu, Dr. Stephen D. Ross, Dr. Maurice Lalonde, Dr. Stanley L. Krugman, Dr. Edward G. Kirby, Dr. J.-André Fortin.

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CONFÉRENCIERS INVITÉS

INVITED PAPERS

PROTOPLAST TECHNOLOGY AND APPLICATIONS TO FOREST SPECIES: WHERE DO WE GO FROM HERE?

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ABSTRACT

A review of the current methods for the isolation and culture of protoplasts of forest species is presented. The role of protoplasts in the development of new procedures in tree breeding are evaluated. Potential uses of somatic hybridization and somaclonal and gametoclonal variation and cytoplasmic and asymmetric hybrids are discussed.

Additional key words: Asymmetric hybrids, biotechnology, cell fusion, conifers, cytoplasmic hybrids, gametoclonal variation, somaclonal variation, tissue culture.

RÉSUMÉ

L'auteur passe en revue les méthodes courantes pour isoler et cultiver les protoplastes d'espèces forestières. Il évalue le rôle des protoplastes dans le développement de nouvelles procédures d'amélioration des arbres. Il discute des emplois possibles de l'hybridation somatique, de la variation somaclonale et gamétoclonale et des hybrides asymétriques.

Mots-clés additionnels: Hybrides asymmétriques, biotechnologie, fusion cellulaire, conifères, hybrides cytoplasmiques, variation gamétoclonale, variation somaclonale, culture des tissus.

INTRODUCTION

New techniques in biotechnology fundamental to plant genetic engineering hold particular promise for the improvement of forest tree species (Karnosky, 1981; Kirby, 1982; Ahuja, 1984; Libby and Rauter, 1984; Powledge, 1984; Torrey, 1985; David and David, in press). Biotechnological procedures with direct application to tree improvement include mass clonal propagation, *in vitro* selection utilizing both natural variation and variation induced in culture, somatic hybridization, somaclonal and gametoclonal variation and genetic transformation using suitable vectors. Fundamental to the research fronts in forest biotechnology are successful procedures for the isolation and culture of protoplasts of tree species. This paper will focus primarily on recent advances in protoplast research with trees and will discuss long range applications to tree breeding.

THE ISOLATION AND CULTURE OF PROTOPLASTS FROM TREE SPECIES

1. Starting Material

Protoplasts have been isolated from a number of forest species; however, there are a limited number of reports of successful protoplast culture (Table 1). Physiological condition of the starting material from which protoplasts of tree species are isolated appears to be a limiting factor affecting both isolation efficiency and ability of protoplasts to be cultured. In isolating protoplasts from cotyledon materials of both Douglas-fir (*Pseudotsuga menziesii*) and maritime pine (*Pinus pinaster*), culturing embryos or cotyledons prior to protoplast isolation significantly improves efficiency of isolation and affects regeneration of dividing cells from protoplasts (Kirby and Cheng, 1979; David and David, 1979; Kirby, 1980; David *et al.*, 1982). Important factors in this culture period prior to protoplast isolation include auxin and cytokinin levels in the medium as well as the level and source of reduced organic nitrogen.

Table 1

Isolation and culture of protoplasts of forest species

Species	Reference
Biota orientalis	David *et al.*, 1981
Copaifera multijuga	Venketeswaren & Ghandi, 1980
Leucaena leucocephala	Venketeswaren & Ghandi, 1980
Picea excelsa	Stermen & Ciesla, 1981
Pinus contorta	Hakman & Von Arnold, 1983
Pinus pinaster	David & David, 1979
Pinus taeda	Teasdale & Rugini, 1983
Populus tremula	Ahuja (personnal communication)
Pseudotsuga menziesii	Kirby & Cheng, 1979; Kirby, 1980
Sapium sebiferum	Venketeswaren & Ghandi, 1980

The isolation of active protoplasts from conifer cell suspension cultures, including loblolly pine (Pinus taeda) (Teasdale and Rugini, 1983) and lodgepole pine (Pinus contorta) (Hakman and Von Arnold, 1983), requires that cell cultures be in the exponential phase of growth at the time of protoplast isolation. Supporting these observations are preliminary studies from our laboratory indicating that at days 4 through 8 after subculture (early exponential phase of growth), cell cultures of Douglas-fir maintained on a medium containing 30 mM. glutamine are capable of yielding large numbers of metabolically active protoplasts.

2. Protoplast Isolation and Purification

Commercially available cell wall degrading enzymes, including both cellulases, hemicellulases and pectinases, have been utilized for isolating tree protoplasts. Many enzyme preparations have been reported as beneficial, normally without the need for purification or desalting. In isolating protoplasts from maritime pine cotyledons, however, an increase in yield and viability was obtained by using desalted pectinase and hemicellulase in combination with non-desalted cellulase (David et al., 1984). In our experience with protoplasts isolated from both cotyledons and cell cultures of Douglas-fir, it has not been necessary to desalt protoplasting enzymes.

Other factors that play significant roles in the isolation of tree protoplasts capable of wall regeneration and division include the nature of the plasmolyzing agents utilized. Most frequently, sorbitol is used as the sole plasmolyzing agent. However, mannitol, either alone or in combination with sorbitol, and glucose have also been used successfully (Redenbaugh et al., 1981; Teasdale and Rugini, 1983; David et al., 1982). Buffering components, including MES (2-[N-morpholino] ethanesulfonic acid), have been reported as beneficial for the isolation of pine protoplasts (David et al., 1984). Incorporating elevated levels of calcium chloride (usually in the range of 2-10 mM) has also been shown to help maintain protoplast integrity. Teasdale and Rugini (1983) report that potassium chloride (0,7 mM) is also beneficial in this regard.

The time required for the release of protoplasts by cell wall degrading enzymes differs with the species utilized, the nature of the starting material (ie. leaf or cotyledon vs. cell culture or callus) and the enzymes employed. Protoplasts can be produced in 3.5 hrs from Douglas-fir cotyledons using high concentrations of enzymes (up to 2.5%, w/v) (Kirby and Cheng, 1979). Lower enzyme concentrations (as low as 0.2%, w/v) required longer incubation periods (up to 24 hrs), as has been reported for cotyledon protoplasts of maritime pine (David et al., 1982) and leaf and cell culture protoplasts of elm (Reddenbaugh et al., 1981).

After protoplasts have been enzymatically released, it is necessary to remove the enzymes prior to culture. This is most frequently done by repeated centrifugation and resuspension, usually in culture medium. Centrifugation procedures, however, frequently disrupt fragile protoplasts. Simple sedimentation, as reported for loblolly pine (Teasdale and Rugini, 1983), is the preferred technique.

3. Protoplast Culture

From recent reports of regeneration of dividing cells from protoplast of tree species, it appears that the culture procedure, although critical, is secondary in importance to the selection of proper starting materials. There are, however, several features that are critical to production of actively dividing cells from protoplasts. Protoplast culture media are based on cell culture media and contain basal medium components, auxin and cytokinin, sucrose or glucose, vitamins, organic supplements and basic amino acids, such as asparagine, glutamine and arginine. Our studies with Douglas-fir protoplasts (Kirby, 1980) demonstrated that a limiting factor in obtaining dividing cells from protoplasts was the presence of glutamine in the culture medium. We also showed that glutamine can substitute for undefined organic medium supplements, such as malt and yeast extracts, or casein hydrolysate. In Douglas-fir the optimal level of glutamine was determined to be 50 mM. Results with protoplasts of other woody species have shown that the glutamine requirement may be universal. In fact, all reports of protoplast cultures of conifers have shown that the addition of glutamine between 10 and 50 mM is beneficial (refer to Table 1 for references).

Other factors may limit the success of protoplast culture. Of primary concern is plating density. A review of the recent literature reveals that plating densities for protoplasts of tree species vary considerably, from 5×10^4 protoplasts per ml in loblolly pine (Teasdale and Rugini, 1983) to 2×10^6 protoplasts per ml in maritime pine. An additional concern is the plasmolyzing agent used in the culture medium, i.e. sorbitol, mannitol or glucose, as discussed above. The culture technique can greatly affect obtaining good cell cultures from protoplasts. The most frequent procedure employed with tree protoplasts is liquid culture. In this procedure, protoplasts are suspended in culture medium at a desirable density and then cultured as small droplets (David *et al.*, 1982), or as cell suspensions, either with or without moderate agitation (Teasdale and Rugini, 1983). We have investigated the use of a thin polyester fleece for support of protoplasts during culture (Kirby, 1980). This material may be beneficial, since it allows free diffusion of inhibitory substances, including phenolics, away from living cells and provides a large surface area for diffusion of oxygen into the medium. In addition, this material forms a matrix within which protoplasts may grow independent from each other. We have obtained excellent results using this material. Recent experiments in our laboratory have shown that high purity low melting point agarose (e.g. *SeaPlaque* from the FMC Corp., Marine Colloids Div., Rockland, Maine, USA) at low concentrations can provide an adequate agar medium for plating of conifer cells and protoplasts.

Regeneration of rapidly growing cell cultures from protoplasts is greatly facilitated by sequential lowering of the osmotic potential of the culture medium. This is achieved in liquid cultures by first allowing protoplasts and cells to settle to the bottom of the culture vessel and then removing a known volume of the original culture medium and replacing it with a new medium of lower osmotic potential. The frequency with which this is done must be determined empirically. Lowering the osmotic potential as frequently as every three days can produce cultures with numerous colonies within three weeks (Teasdale and Rugini, 1983).

APPLICATION OF PROTOPLAST TECHNIQUES TO TREE IMPROVEMENT

1. Somaclonal and Gametoclonal Variation

While most of the initial excitement surrounding the development of plant protoplast techniques centered of their potential use in somatic hybridization and production of novel hybrids, the most rewarding application of protoplasts to date has been their use in somaclonal variation. Somaclonal variation is simply defined as variation detected in plants derived from any form of cell culture (Evans et al., 1984). Correspondingly, gametoclonal variation is variation detected in plants derived from cultures of gametophytic (haploid) tissues. Protoplasts are particularly useful in applications of somaclonal variation because they may be innately more genetically susceptible to variation induced in vitro, but also because techniques enabling regeneration of whole plants from single protoplasts can insure that an individual regenerated plant will be genetically uniform, that is, by using protoplasts, the incidence of chimeras can be greatly reduced. Applications of somaclonal variation have been reported for a number of economicaly important species including carrot, geranium, tobacco, tomato, alfalfa, sugar cane, wheat, rapeseed, pepper, rice, oats, corn and potato (Larkin et al., 1983; Evans et al., 1984).

There is growing evidence that somaclonal variation is a consequence of specific genetic events that take place during the early initiation of plant cell cultures. Many types of variation are induced in culture, including polyploidy, aneuploidy, mitotic irregularities (including structural changes and mitotic recombination), single gene nuclear mutations and changes in cytoplasmic DNA. Perhaps one of the most exciting features of somaclonal variation is the frequency of induced variability. The frequency of single gene mutations in tomato can be as high as 1 mutant in every 20-25 regenerated plants (Evans et al., 1984).

Currently, investigators are making use of variation which is induced in culture in producing improved varieties of horticultural and agronomic crops. The application of somaclonal variation to tree breeding is certainly forthcoming as new procedures become established which allow routine regeneration of plants from protoplasts and cell cultures. Researchers working with tree species, however, must appreciate the tremendous genetic variability existing within natural populations of the organisms with which they work. Considerable effort should be focused on development of new screening procedures that attempt to capture natural variability for specific breeding purposes.

2. Somatic Hybridization

Although somatic hybridization is potentially an extremely powerful technique, application of cell fusion technique is far from routine. There are many reasons for this, including cytoplasmic "incompatibility" in hybrids and difficulty in obtaining synchrony of the cell cycle of fusion partners prior to fusion, resulting in chromosome elimination during subsequent cell divisions. In spite of these problems, most of the recent research has centered on transfer of cytoplasmically encoded characteristics or transfer of a limited number of nuclear genes for which adequate selection systems have been established (Evans, 1983; Pelletier and Chapeau, 1984).

Procedures for protoplast fusion first involve the preparation of active protoplasts followed by either chemically or physically induced fusion. The polyethylene glycol (PEG) method for fusion (Kao and Michayluk, 1974) has produced consistently good results with a variety of species. In this technique, freshly isolated protoplasts are exposed to a solution containing 25-40% PEG (1600-6000 mw), which causes agglutination of the protoplasts. The PEG solution is then eluted with a solution containing high levels of calcium and a high pH. This procedure has resulted in fusion frequencies as high as 100% (Vasil _et al._, 1975). Fused protoplasts are then cultured on a liquid medium, cell walls are regenerated, cell division takes place and plants are regenerated.

An alternative to the use of PEG for protoplast fusion is the use of electric field induced fusion (electrofusion) (Gaynor, in press). Electrofusion first involves close alignment of protoplasts by application of a non-uniform alternating electric field. Fusion results when a brief pulse of high voltage direct current is applied (Zimmerman _et al._, 1976; Zimmerman, 1982). A recent report (Kohn _et al._, 1985) describing electrofusion of protoplasts derived from complementing nitrate reductase-deficient cell lines of tobacco followed by regeneration of somatic hybrid plants indicates that further application of electrofusion in obtaining hybrids will be made. Advantages of electrofusion include synchronous aggregation and fusion. In addition, duration of the fusion pulse can determine the actual number of protoplasts which fuse. In contrast to PEG-induced protoplast fusion, electrofusion appears to offer much finer control of the fusion process.

Cell cultures derived from fusion experiments consist of heterogeneous populations of parental cells, homokaryotic fusion products and heterokaryotic fusion products. It is necessary, therefore, to establish a definitive procedure for selection of heterokaryotic fusion products. This can be accomplished by the use of genetic markers, which may involve specific growth features characteristic of the hybrids and not of the parentals. An alternative procedure, and one which has had far wider use, is microselection. In microselection, fusion products are visually identified and cultured independent from other cells (Evans, 1983). Callus cultures are then produced followed by regeneration of the hybrid plants using standard techniques.

3. Cytoplasmic and Asymmetric Hybrids

The production of true somatic hybrid plants may be a formidable task, relying more on good fortune than on sound technique. Cell fusion procedures, however, are being used at present to produce unique genetic combinations otherwise difficult to achieve. Cytoplasmic hybrids (cybrids) which involve substitution of one cytoplasmic genome for another, have been characterized for a number of species (Pelletier and Chapeau, 1984). This technology holds particular promise in the production of new male sterile genotypes for important crop plants and, perhaps, forest species (Hanson, 1984). The production of asymmetric hybrids, in which only partial transfer of the nuclear genome is achieved, offer an additional application of fusion technology. It is possible to transfer limited genes via fusion of normal and highly irradiated protoplasts (Gupta et al., 1984). Further applications of asymmetric fusions await development of suitable selection systems.

CURRENT LIMITATIONS OF PROTOPLAST TECHNOLOGY

As to the current breeding applications of somatic hybridization, limitations do exist. With the exception of the production of specific male sterile lines of tobacco, no new varieties of plants have been produced using cell fusion technology (Cocking, in press). In order to facilitate the use of the protoplasts in breeding, efficient systems for regeneration of plants from protoplasts must be developed. At present, relatively few plants can be regenerated from protoplasts and there are no reports of reliable regeneration of forest trees from protoplasts. In addition, if somatic hybrid plants are to be useful in breeding programs, they must be capable of efficient sexual crossing. Problems that have been encountered in obtaining fertile somatic hybrids may be able to be overcome if mitotic recombination between parental chromosomes were somehow encouraged in fusion products prior to the critical period of chromosome elimination. In addition, it may be necessary to manipulate the chromosome number in hybrid cell lines or plants in order to obtain fertile plants.

Perhaps, potentially the most useful contribution of protoplast and cell culture technology to forest genetics lies in the area of somaclonal variation, where specific traits can be efficiently recognized in variant plants regenerated from somatic cells and protoplasts. By placing particular emphasis on selection of monogenic and allelogenic traits, such as disease resistance and herbicide tolerance, extremely desirable phenotypes of forest trees can be generated. There remains, however, a formidable challenge to basic researchers: we must establish an efficient and predictable method for regeneration of trees from cell cultures before many of the advances in crop biotechnology may be applied to forest species.

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STATUS OF FLOWERING IN CONIFERS: A CONSTRAINT TO TREE IMPROVEMENT?

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ABSTRACT

For Pinaceae family conifers we now have the ability to circumvent, by appropriate hormonal (giberrelin $A_{4/7}$ mixture) and cultural (girdling, modest water stress, root-pruning, NO_3-N fertilization, high temperature) treatments, the biological constraints posed by sexual juvenility. Problems of flowering potential and periodicity also no longer need constitute a major obstacle to the breeding and production of genetically improved seed. Realization of this potential, however, requires a change in our traditional approach to seed production-- i.e., in soil-based orchards comprised of large, widely spaced trees, with little control over climatic and treatment factors, or pollen parentage. Alternative seed production systems are considered. Of these, indoor-potted orchards at present appear to provide the best opportunity for rapidly, completely and economically realizing the benefits of tree improvement in reforestation.

RÉSUMÉ

Pour les conifères de la famille des Pinacées, nous avons maintenant les moyens de circonvenir les contraintes posées par la juvénilité sexuelle: ces traitements appropriés sont de nature hormonale (mélange de gibberelline $A_{4/7}$) et de nature culturale (cernage, stress hydrique modéré, taille des racines, fertilisation au NO_3-N, température élevée). De même, les problèmes de potentiel de floraison et de périodicité ne constituent plus un obstacle majeur à l'amélioration et à la production de semence génétiquement améliorée. Cependant, la réalisation de ce potentiel nécessite un changement dans notre approche traditionnelle de la production de semence, c'est-à-dire dans des vergers sur sol comprenant de gros arbres largement espacés, sans grand pouvoir sur les facteurs du climat et les traitements ou sur la parenté pollinique. On considère des systèmes "alternatifs" de production de semence. Parmi ceux-ci, les vergers en pots sous abri semblent pour le moment offrir la meilleure occasion de réaliser rapidement, complètement et économiquement tous les bénéfices que l'amélioration des arbres apporte en reboisement.

INTRODUCTION

Much has been written in recent years about the inefficiencies of propagating conifers by sexual means (e.g., seed), especially in relation to the potential of clonal forestry (e.g. Libby, 1985). The future role of sexual propagation in tree improvement is increasingly being questioned. For most conifers, however, the "potential of clonal forestry" is simply that-- a potential whose possible realization will require many additional years of further research, testing and validation. Additionally, unless there are breakthroughs in the economics of tissue culture propagation, or somatic embryogenesis propagation can be shown to be truly clonal (and feasible), the number of propagules produced from any one "superior" genotype will probably remain limited, especially if those propagules are derived from tested mature genotypes and families. Furthermore, propagules derived from such mature genotypes (as opposed to young seedlings) will have many of the characteristics of mature/maturing trees, including slower growth (Sweet and Wills, 1974; Greenwood, 1984). Sexual reproduction will still probably be the most efficient way to create the new variation upon which future genetic improvement must depend. And, if the economics and dependability of seed production are improved, it may remain the most viable option for reforestation with superior genotypes. This does not preclude, however, a vegetative propagation program (traditional or tissue culture) being combined with an economically improved seed production program (Smith _et al._, 1981), especially if early progeny testing becomes feasible (Pharis and Ross, 1985a,b).

Rather than waiting for the new "biotechnology of vegetative propagation" to replace propagation of our forests by seed/seedlings, we should be considering what can, in fact, be done now to improve the efficiency of seed production. The biological and practical constraints to flowering in forest trees have been the subject of numerous reviews (Jackson and Sweet, 1972; Puritch, 1972; Lee, 1979; Ross and Pharis, 1982, 1985; Pharis and Ross, 1985a; Owens and Blake, 1985) and several recent symposia (Bonner, 1979; Krugman and Katsuta, 1981), including two in 1985 (see Pharis _et al._, 1985 and Ross _et al._, 1985b). Here we will only attempt to briefly summarize the progress to date in overcoming these constraints to breeding and seed production in conifers.

BIOLOGICAL CONSTRAINTS

Juvenility, The Problem

Hackett (1985) has defined juvenility as that period during the early ontogeny of most woody perennial plants grown from seed when flowering does not occur naturally and cannot be induced by the normal floral-initiating treatments. Using age to first flowering in natural stands as a criterion, the juvenile phase may last from only a few years (e.g. Pinus contorta Dougl. and P. banksiana Lamb.) to 30 or more years (e.g. some species of Abies and Picea). Typically, though, the so-called juvenile phase is 10 to 20 years for most north temperate conifers (Puritch, 1972; Hackett, 1985; Zimmerman et al., 1985).

However, as trees may not flower for some years after sexual maturity is attained, presumably because climatic and site conditions are not favorable, this age to first flowering can be misleading. Ross et al. (1983) cite the example of seedling seed orchards of Pseudotsuga menziesii (Mirb.) Franco established on southern Vancouver Island, both on cool, wet locations and also in the relatively sunny rain shadow of the Saanich Peninsula, near Victoria. Flowering in any one family at the latter location may begin (and continue) after only 3 to 5 years of age, 5 to 10 years earlier than flowering in the cool, wet location. Is then "sexual maturity" of P. menziesii reached at age 3 years, as evidenced by this early flowering on this one rather unique site within its normal range? A survey over the vast majority of sites throughout its normal range would set the age of "sexual maturity" in excess of 10 to 15 years (Puritch, 1972). Because of anomalies such as this, Zimmerman et al. (1985) have produced a somewhat modified version of when phase change occurs:

> "Phase change has occurred (1) if a plant flowers, no matter how flowering is induced, and (2) if the said plant will then continue to flower in its natural environment without application of any artificial stimulator that may have originally induced flowering".

This latter definition seems to us to best fit our current knowledge of natural controls over flowering, and our knowledge of just how those controls can be circumvented.

Juvenility is a concern that has yet to be faced and dealt with by most tree improvement programs here in Canada or elsewhere. For the most part, breeding and seed production activities are still being carried out with ramets of phenotypically "superior" adult trees whose sexual maturity is maintained, more or less, in vegetative propagation. There may be a temporary suspension of flowering in these "mature" scions following rooting or grafting onto seedling rootstock and this may superficially resemble the juvenile condition of young seedlings. However, the former can often be readily stimulated to flower by the usual floral-initiating cultural treatments (water stress, girdling, root-pruning, high temperature, N fertilization), whereas very young seedlings normally will not (see Puritch, 1972; Hackett, 1985; Ross and Pharis, 1985).

Juvenility, furthermore, should not become a practical problem so long as forest geneticists retain a conservative approach to progeny testing, waiting 10 to 15 years before selecting the next-generations's breeding and seed production populations. By that age the selected ortets (and their vegetatively propagated ramets) should have already attained a significant degree of sexual maturity, although the vagaries of climate and poor choice of site may still limit the practical production of seed, even from so-called "sexually mature" orchards (more on this later). However, the future should see a substantial shortening of the testing period. Lambeth (1980) has demonstrated the reliability of early selection for growth traits in Pinus taeda L. and some other conifers based on 4- to 6- year progeny performance in well designed field tests. New approaches to physiological testing under controlled-environment glasshouse conditions (see examples cited in Ross et al., 1983 and Pharis and Ross, 1985b) may some day enable identification of superior individuals and families at less than a year of age.

To better comprehend the possible impact of juvenility on future breeding and seed production programs, it is necessary to consider the phenomenon called "phase change" in more detail. Some workers (Borchert, 1976; Pharis, 1977; Greenwood, 1981; Ross et al., 1983) question whether conifers exhibit a true juvenile phase with regard to flowering, suggesting that the normal reproductive incompetence of young seedlings is associated with other endogenous causes that are themselves under phase-change control.

As a tree matures its growth rate declines, in part due to increasing morphological complexity and competition among meristems (i.e. ageing), but also reflecting a nonreversible maturation effect that is retained in vegetative propagation (Sweet and Wills, 1974; Greenwood, 1984). Greenwood (1981) concluded that young P. taeda seedlings normally do not flower because, relative to older trees, their shoots continue growing too long into autumn to set a terminal bud capable of differentiating reproductively prior to the onset of winter dormancy. Such seedlings can be induced to set bud early by a treatment known as "out-of-phase dormancy", and this treatment often also causes precocious flowering (Greenwood, 1981).

Most of the cultural practices which promote flowering in conifers (drought, girdling, root-pruning, etc.) also retard shoot elongation (see Ross and Pharis, 1985). Several of these floral-inducing treatments have been shown to retard the metabolism of the less polar [^{3}H] GA$_4$, and to increase the level of less polar GAs which are relatively low in nonflowering control trees (see references cited in Ross et al., 1983; Pharis and Ross, 1985a). Applied exogenously, certain of the less polar GAs (GA$_4$, GA$_7$, GA$_9$) are highly effective in promoting flowering in Pinaceae family conifers, where they also stimulate shoot elongation especially in younger individuals. A working hypothesis is that young, vigorously growing trees utilize endogenous GAs preferentially for vegetative growth. Only when maturation, environmental or cultural treatment factors restrict vegetative growth do endogenous less polar GAs become available for a long enough time (during the conebud differentiation period) at sufficiently high concentrations for sexual differentiation to occur.

The traditional concept of phase change based on herbaceous and woody angiosperms may no longer be strictly applicable with respect to flowering in conifers. The definition put forward by Zimmerman et al. (1985) appears more likely, as does the maxim, 'the older the tree, the greater its ability to flower'. However, it does seem that there is no true age constraint to flowering in conifers, provided the appropriate stimulus is applied at the proper time of shoot ontogeny. For most conifers it appears that the appropriate stimulus includes GAs.

Overcoming the Strictures of Juvenility

It is well known that for many conifers of the Cupressaceae and Taxodiaceae families, a variety of GAs when applied exogenously will cause profuse flowering even in very young seedlings (see ref. cited in Pharis and Kuo, 1977). Conifers of the Pinaceae family respond, but generally only to certain of the less polar GAs, most notably a mixture of GA_4 and GA_7 ($GA_{4/7}$). Pharis and Ross (1985b) cite nearly 80 referenced reports which demonstrate the effectiveness of exogenously applied $GA_{4/7}$ for promoting flowering in at least 16 species representing 5 of the 6 genera of this commercially important family.

For reasons that are not completely clear, seedling members of the Pinacese family as a group are not as responsive to applied GAs as are those of the Cupressaceae and Taxodiaceae (Pharis and Ross, 1985a). Also, the $GA_{4/7}$-induced flowering in Pinaceae family conifers is much more dependent upon the use of adjunct cultural treatments (e.g. root-pruning, water stress, girdling, N fertilization, etc.), which may be ineffective by themselves but can often enhance synergistically the flowering response to GAs (see Ross and Pharis, 1982, 1985). Although these cultural and $GA_{4/7}$ treatments do work best on more mature individuals they can still be highly effective on young, presumably still "juvenile", seedlings.

Tsuga heterophylla (Raf.) Sarg. is a good illustration of this. Within its natural range this species has one of the longer juvenile phases of any conifer (20-25 years). Yet, it is possible to induce profuse flowering in container-grown seedlings only 2 years old from seed when $GA_{4/7}$ is applied in conjunction with nitrate-N fertilization, high temperatures and (or) water stress (Pollard and Portlock, 1981; Brix and Portlock, 1982). *Pseudotsuga menziesii* is another Pinaceae family conifer with a relatively long juvenile phase in nature (10-20 years) for which similar treatments will cause precocious flowering, the earliest thus far being three years of age (S.D. Ross, unpublished results). Thus, when $GA_{4/7}$ is used in conjunction with the appropriate cultural conditions (which appear to often be species dependant) there is every reason to believe that flowering in most conifers can be promoted at a very early age.

Hastening Sexual Maturation--Is It Possible?

It is important to emphasize that even though applied GAs can be used to induce precocious flowering in conifers, they only over-ride and do not permanently terminate the juvenile condition associated with young seedlings (see Zimmerman *et al.*, 1985). Without subsequent retreatment a seedling thus induced might not again flower until sexual maturity was naturally attained several years later. Fortunately, we have at our disposal a practical method which appears, for all practical purposes, to accelerate the sexual maturation process.

It is now well established that for many woody angiosperms the attainment of the adult condition is primarily a function of tree size rather than age per se (see Hackett, 1985). It may be than an angiosperm seedling must first attain a certain minimum size before it can flower, and it makes little difference whether this size is achieved through continuous growth or the normal succession of annual growth increments. In conifers as well we find that growing seedlings under extended photoperiods in a heated greenhouse prior to outplanting in the seed orchard can considerably shorten the age to first flowering (Young and Hanover, 1976; Wheeler *et al.*, 1982; Hackett, 1985).

This may seem paradoxical, given our assumption that the normal reproductive incompetence of young conifer seedlings is associated with their inherently vigorous vegetative growth. However, J.E. Webber (personal communications) has found some caveats for *Picea glauca* (Moench) Voss. In comparison with "non-accelerated" control plants, accelerated-grown seedlings only flowered earlier if they were then exposed to appropriate stress conditions, such as drought, which restrict vegetative growth. A similar stress situation may be occurring under the field conditions cited by Young and Hanover (1976) and Wheeler *et al.* (1982).

Growth acceleration of conifer seedlings may hasten the sexual maturation of individual apical meristems as appears to be the case for some woody angiosperms (Hackett, 1985), although there is no direct evidence for this with conifers. Or, as previously discussed, the effect of maturation may be indirect through a reduction in the shoot's growth potential. The growth-acceleration treatment also promotes branchiness (Wheeler *et al.*, 1982), so that more shoots presumably also will be "maturing" in a given period. In the study of Wheeler *et al.* (1985), *P. contorta* seedlings grown under continuous light for 6 months in a heated greenhouse prior to outplanting differentiated seed and pollen cones, respectively, at 2 and 4 years of age. This was one year in advance of nonaccelerated-grown seedlings on the same site. By age 5 years, the accelerated seedlings were 61% taller and had 79% more branches than the controls. And, the following year they initiated, on average, 6.1 and 5.5 times as many seed- and pollen-cone buds, respectively. Hence, their increased flowering response may reflect both direct "maturational" effects as well as indirect "having more branches" effects. Or, the larger, branchier accelerated-grown seedlings may be under greater water stress from the mere logistics of supplying a larger shoot from approximately the same volume of soil. If this were the case, then the flowering response to accelerated growth would not involve increased "maturation" at all.

To reiterate Hackett (1985), "the best formula which can be given to obtain rapid flowering in many tree species is to grow the seedlings as rapidly as possible to a certain species, dependent size and then apply the flower-inducing treatment which is appropriate for the species". This strategy of early growth acceleration followed by $GA_{4/7}$ plus an appropriate cultural treatment has now been successfully used to promote very precocious flowering in several Pinaceae family conifers (Ross, 1978; Greenwood, 1981; Brix and Portlock, 1982; Cecich, 1983).

Limitations Placed on Flowering by the Number of Potential Conebud Differentiation Sites

The concern is frequently expressed that seed yields per tree and per hectare will necessarily be lower for future advanced-generation orchards comprised of younger, sexually immature selections. However, to a large extent the age-dependent increase in flowering probably has less to do with age or maturation than with the number of potential flowering sites. Thus, we observe for Pseudotsuga menziesii what is probably a characteristic of conifers in general. A clonal seed orchard may come into flowering (naturally or after cultural and (or) $GA_{4/7}$ treatment) earlier, but the seedling seed orchard rapidly catches up and soon becomes much more productive (Konishi, 1985). The fact is that seedlings develop potential flowering sites much more rapidly than do vegetative propagules of mature trees. This is reflected not only in the inherently faster growth rate of seedling-origin trees, but also their greater branchiness as measured by number of lateral shoots per unit length of stem (Sweet and Wills, 1974; Wheeler et al., 1982; Greenwood, 1984).

It would be a mistake, however, to equate flowering potential simply with number of shoots. Even for mature trees, and even in years conducive to abundant flowering, only a relatively small proportion of the total shoots may be contributing to female or male flowering. When one considers also the number of axillary and (or) terminal apices per shoot that are potentially capable of differentiating reproductively, we find that the realized potential for flowering in most Pinaceae family conifers is very low indeed (Owens and Blake, 1985).

Conifers generally exhibit a pattern of sexual zonation whereby seed cones are concentrated in the upper crown and pollen cones in the lower crown, with a transition zone in between where cones of both sex may be produced, often on the same shoot (Pharis and Morf, 1968; Marquard and Hanover, 1984). These zones may be extended but the pattern still holds even under conditions (natural and artificially induced) highly favorable for flowering (Chalupka, 1981; Ross *et al.*, 1981; Marquard and Hanover, 1984). However, whether a shoot differentiates mainly seed cones, mainly pollen cones, or both, appears to depend relatively more on its vegetative vigor than on its position *per se* within the crown. The following relationship between increasing shoot vigor and probable bud developed described by Tompsett (1978) for *Picea sitchensis* (Bong.) Carr. seems to hold but with slight modification for many Pinaceae family conifers: weak vigor = vegetative pollen cone, intermediate vigor= vegetative seed cone, strong vigor = vegetative.

Sweet and Krugman (1978) proposed crown pruning as a means not only of making cones more accessible from the ground, but also for redirecting the otherwise rapid height growth into increased production of lateral shoots with a high flowering potential. Ross and Pharis (1982) review a number of studies on conifers which demonstrate the practicality of top pruning for achieving both of these objectives. Here, increased cone production frequently resulted, even though the method of pruning was not designed to specifically favor those types of shoots most predisposed to differentiate conebuds. Also, top pruning in these studies was usually delayed until the seed orchard trees became too tall for efficient management. From the experience with fruit tree orchards (Jackson, 1985), pruning beginning at a relatively young age would seem to provide the best opportunity for manipulating crown architecture to maximize potential flowering sites, especially if, at the same time, trees are optimally cultured for rapid vegetative development.

Flowering Periodicity

In well-managed seed orchards, as in nature, even mature trees exhibit considerable periodicity in flowering. Thus, most trees initiate few if any conebuds for one or possibly several years following a heavy cone crop, and, depending on the site and species, the interval between years of abundant flowering is generally much longer.

Studies correlating seed crops in conifers with weather data (e.g. Rehfeldt *et al.*, 1971; Eis, 1973) indicate that the proper sequence of optimal environmental conditions (e.g., dry or even droughty, with high solar insolation, during the late spring or summer prior to initiation/differentiation of conebuds) for flowering may occur, but infrequently, in nature. However, for a variety of reasons (see Rehfeldt *et al.*, 1971; Owens and Blake, 1985), such studies do but a poor job of quantifying the optimal environmental conditions for any given stage of reproductive bud development. This information is now slowly becoming available through growth-chamber and greenhouse studies using container-grown trees (Pollard and Portlock, 1981; Longman, 1982; Philipson, 1983; Ross, 1985).

This information on optimal environmental conditions for conebud initiation/differentiation can be profitably used to select field seed orchard sites with a high probability of consistently providing abundant flowering and subsequent good cone development. Cultural practices can also be used to moderate, though not totally eliminate, the natural periodicity in flowering (see Wheeler *et al.*, 1985). Even the most effective of these practices, even when applied together with GAs, will not compensate for an otherwise unfavorable (usually wet and cool, with low solar insolation) environment (Dunberg, 1980; Ross and Pharis, 1982).

Owens and Blake (1985) emphasize that there is also an endogenous component to the periodicity in flowering in sexually mature trees which determines how frequently the tree should be given cultural and (or) GA$_{4/7}$ treatments for best results. Wheeler *et al.* (1985) recently reported the successful promotion of flowering in 4 consecutive years from annual stem girdling in grafted *Pseudotsuga menziesii* seed orchards. However, it was only in the last year of their study that the cone crop induced could be considered heavy in relation to tree size. It is doubtful that the girdling or other treatments would have been effective in overcoming the bienniality of flowering which normally follows such a heavy cone crop year. Developing cones and seeds constitute a strong sink for nutrients (Dickmann and Kozlowski, 1970), and their period of maximum growth in many conifers coincides with the differentiation of lateral primordia, a weak sink, into reproductive cone buds (Owens and Blake 1985). Also, because conebuds differentiate at the expense of vegetative buds (Pharis *et al.* 1980), prolific flowering one year may significantly reduce the number of shoots with lateral primordia that are capable of differentiating reproductively the following year (Owens and Blake, 1985). This was particularly apparent with potted *Picea engelmannii* grafts that had been induced to flower profusely by the highly effective combination of properly timed heat, drought and GA$_{4/7}$ treatments (Ross, 1985 and unpublished results).

Furthermore, it must again be emphasized that most of the cultural practices used to promote flowering in conifers are also stress treatments (Ross and Pharis, 1985). Consequently, their repeat application in consecutive years will further retard vegetative development and thus the recovery of flowering potential, as well as adversely affecting the development of the existing pollinated conebuds and their seeds (S.D. Ross, unpublished results). Our results for *T. heterophylla* and *P. engelmannii* (Ross *et al.*, 1985b) indicate that the optimal strategy for maximizing cone production in the long term is to apply promotion treatments every other year (and then no longer nor more severely than absolutely necessary), keeping trees well watered and fertilized inbetween to ensure rapid recovery of vegetative tissues and normal cone and seed development in the off-treatment year.

Clonal differences

The so-called "20/80" rule was coined by one southern pine tree improvement cooperative (Anon., 1976) to reflect the fact that 80% of its orchard seed were produced by only 20% of the clones. The ratio can actually be much more disproportionate than this, especially in young orchards and on sites and years unfavorable for flowering (Eriksson, 1978; Wheeler _et al._, 1985). However to our knowledge, a strong negative genetic correlation between fecundity and vegetative growth rate has not been shown (see Libby, 1985), although this does not mean it does not exist. Of more serious concern are the negative impacts of these clonal differences in fecundity on seed orchard yields and genetic efficiency (Eriksson, 1978; Smith and Adams, 1983; Wheeler _et al._, 1985).

Although our cone-enhancement treatments are almost always most effective on those clones (and families) that are inherently predisposed to flower, they still may be profitably used to increase the proportion of genotypes flowering and to dampen the differences in fecundity among them (Pharis _et al._, 1980; Ross _et al._ 1981, 1985b). Wheeler _et al._ (1985) reported that stem girdling in a grafted _Pseudotsuga menziesii_ seed orchard doubled both the proportion of ramets and clones producing seed cones, and that pollen density was four times as heavy in the girdled as in the ungirdled portion of the orchard.

Ross _et al._ (1985a) compared 10 each of previously good- and poor-flowering, half-sibling _P. menziesii_ seed orchard families for their response to $GA_{4/7}$ and root-pruning. Treatments were compared alone and together. In combination, the two treatments had a highly synergistic effect on flowering, and the magnitude of this synergism was significantly greater for the poor- than good-flowering families. Without treatment, 50% of the poor-flowering families flowered (one seed cone bud per tree, on the average) compared to 80% of the good-flowering families (an average of 20 seed-cone buds per tree). When $GA_{4/7}$ plus root-pruning was given together, all 10 families in each group of trees flowered. And, the difference in mean seed-cone buds per tree between the poor- and good-glowering families (413 and 928 each, respectively), although still significant, was also much reduced. A similar "treatment by clone interaction" was also noted for _T. heterophylla_ in response to the combined application of $GA_{4/7}$ + calcium nitrate fertilizer (Ross _et al._ 1981). Probably most clones and families, regardless of their inherent fecundity, are susceptible to flowering promotion if given the appropriate stimulus.

PRACTICAL CONSTRAINTS TO SEED PRODUCTION

The message we have tried to convey in the preceding sections is:

> "treatments are presently available that can be used to overcome many of the biological constraints to flowering,

> at least to the extent where the lack of flowering no longer need constitute the major obstacle to breeding and seed production programs".

However, there are other constraints, including economic ones, with regard to the continued use of the traditional soil-based seed orchards.

Much effort has been devoted to the development and implementation of new technology to overcome the well known production, genetic and management inefficiencies of conventional seed orchards (see Sweet and Krugman, 1978; Ross and Pharis, 1982; Ross *et al.*, 1985b). This includes various methods already discussed to ensure earlier, more reliable flowering and top pruning for size control; supplemental mass pollination for improved seed set and genetic gains (see Wheeler and Jech in this volume); and installation of costly overhead-misting systems for avoidance of contaminating foreign pollen and for protection against frost (El Kassaby *et al.*, 1984). However, it is time that we begin to question the effectiveness, operational practicality, and economics of such remedial actions and consider instead those alternative seed production systems which circumvent the inherent limitations of conventional seed orchards.

Miniaturized Field Seed Orchards

There is much we can learn from the experience of fruit tree orchardists. Jackson (1985) points out that they too started out with large, spreading trees similar to our seed orchards today, but found it was far more efficient to diffuse fruit production among many small, closely spaced trees, using crown pruning both to control size for easy management and to maximize potential flowering sites. Sweet and Krugman (1978) adapted this concept to *Pinus radiata* seed orchards, as have Longman and Dick (1981) for *Thuja plicata* Donn. The approach differs somewhat for the two species, however. With *T. plicata*, Longman and Dick (1981) essentially miniaturized a conventional orchard, with trees planted at very close spacing but with clone members still separated to favor random mating through wind pollination. On the other hand, what Sweet and Krugman proposed for *P. radiata* was in essence a group of 2-clone orchards wherein ramets are grown in low clonal hedges, and in which pollen parentage is controlled through artificial pollination rather than juxtaposition of clones.

Each proponent described the demonstrated advantages of their approach for their particular species and program. Breeding of *P. radiata* in New Zealand is sufficiently advanced so as to be able to capitalize on specific-combining as well as general-combining effects of superior clones through artificial pollination. This, however, was not the case for *T. plicata* in Scotland. Another major difference relates to size control, which again is determined by species characteristics. With *T. plicata*, trees are induced to flower profusely while still quite small by treatment with GA_3, and severe top pruning then follows as a means also for conveniently harvesting the seed cones, which are concentrated in the upper crown (Longman, personal communications). As with most species of the Cupressaceae, T. plicata does not lack for potential flowering sites; however, this plethora of potential conebud differentiation sites is not the case for most Pinaceae family conifers. In Sweet and Krugman's approach with P. radiata, hedge development begins at a young age and has as a major objective increasing the number of potential conebud differentiation sites, after which $GA_{4/7}$ and other treatments may be used to promote early flowering.

Miniaturization of soil-based field orchards by either approach represents an advance over our present approach to seed production. We feel that further testing with other species is definitely warranted. As discussed in the current Members' Report by Ross, preliminary results with early crown pruning of P. menziesii and T. heterophylla are quite encouraging. Seed yields per tree may be less, but many more trees will be contributing to a higher overall production on a per-hectare basis. Conebud enhancement by $GA_{4/7}$ and cultural treatments can also begin earlier in the miniaturized orchard owing to its much shorter period required for early vegetative development, during which induction treatments of a stressful nature are not recommended (see Ross and Pharis, 1982). We have furthermore found that many stimulatory treatments, $GA_{4/7}$ in particular, are much more effectively and efficiently applied with smaller trees.

Indoor-Potted Seed Orchards

Whatever the approach to soil-based orchards, seed production will still be subject to a significant degree to the vagaries of climate and the whims of nature. And, regardless of tree size or clonal arrangement, there remain serious practical difficulties in attempting to control pollen parentage in field-grown trees. Since 1980, the British Columbia Ministry of Forests has been actively involved in the development and evaluation of indoor-potted orchards as a practical alternative to conventional soil-based seed orchards for T. heterophylla and the interior spruces, P. engelmannii and P. glauca. Our progress to date is summarized by Ross in recent Members' Reports, and Ross et al. (1985b) provide a detailed discussion of the many identified (and demonstrated) advantages of working with small, potted trees, subject to intensive management and strict environmental control. These advantages include:

(a) earlier, more reliably abundant flowering, with a greater proportion of clones contributing to seed production;

(b) ease of conebud induction, pollen management, seedcone collection and virtually all other management practices;

(c) an increased ability to protect cones and seeds from adverse climatic conditions and fungal and insect pests that could otherwise, in some years, result in total crop failure;

(d) strict control over pollen parentage for maximum genetic gains;

(e) clonal composition is not fixed but can be rapidly upgraded to include new "progeny-tested" selections as soon as they become available;

(f) more efficient site utilization and the flexibility to rapidly scale production capacity upward or downward to meet changing planting requirements; and

(g) absolute flexibility of siting as, for example, in conjunction with a container nursery where facilities and labor may be shared for increased efficiency, or on inexpensive nonagricultural land not otherwise suitable for cone production.

Ross et al. (1985b) point out that these advantages of indoor-potted orchards can probably be achieved at a cost equal to or even less than that of conventionally produced seed. They give as a hypothetical example two B.C. interior spruce seed orchards, one potted and the other field, each with an annual production capacity of five million viable seeds. The latter field seed orchard would conservatively occupy 3.5 ha of expensive agricultural land and then not attain full production for 15 years after establishment. The potted seed orchard would only require 1 400 m^2 of land, half of that space in relatively inexpensive plastic-covered houses, with full seed production reached in only 7 years. Operating costs promise to be lower as well owing to the greater ease of management and increased efficiency of production brought about by the use of small potted trees.

CONCLUSIONS

We now have at our disposal the knowledge and techniques to largely overcome those biological constraints to flowering in conifers that have long been considered as the major obstacle to the breeding and production of genetically improved trees. This is not to say that further research on the mechanism of flowering and its efficient control is no longer necessary (see Owens and Blake, 1985 for discussion of future research needs). However, what is now needed is a change in the way that we now go about producing our improved seeds (e.g., currently in soil-based orchards comprised of large, widely spaced trees with wind as the agent for pollination). Serious consideration must be given, we think, to alternative seed-production systems which can circumvent many of the well known limitations (production, genetic and management) of such field seed orchards. Miniaturization of soil-based orchards, possibly coupled with artificial pollination, is one such system that may work well for some species and programs if an appropriate climatic location exists. For many other species and regions, however, we believe that the indoor container-grown seed orchards will offer the best potential for rapidly and efficiently realizing the full benefits of tree improvement possible through sexual propagation.

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RECENT ADVANCES IN THE APPLICATION OF BIOCHEMICAL METHODS TO TREE IMPROVEMENT

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ABSTRACT

Recent biochemical studies of forest trees such as enzyme electrophoresis and gas chromatography of terpenes have shown that for most species levels of variation were high, that there was considerable genetic differentiation among conspecific populations, that the patterns of differentiation were complex and often associated with the geography, that forest trees were predominately outcrossers, and that opportunities existed for indirect selection. These results were discussed in relation to their utility in tree improvement.

Additional keywords: population structure, gene conservation, seed zones, breeding zones, multivariate statistics, genetic distance, Pinaceae.

RÉSUMÉ

Des études biochimiques récentes portant sur les arbres forestiers, telles l'électrophorèse des enzymes et la chromatographie en phase gazeuse des terpènes, ont démontré que pour la plupart des espèces, les niveaux de variation sont élevés, qu'il y a une différenciation génétique considérable à l'intérieur de populations conspécifiques, que les modèles de différenciation sont complexes et souvent liés à la géographie, que les arbres forestiers sont surtout des "exogames" et qu'il existe des occasions de sélection indirecte. Ces résultats sont discutés par rapport à leur utilité dans l'amélioration des arbres.

Mots-clés additionnels: structure des populations, conservation des gènes, zones de semences, zones de reproduction, analyse multivariée, distance génétique, Pinacées.

In recent years, the use of biochemical methods for tree improvement purposes has increased rapidly. Two such methods are electrophorectic analysis of molecular differences in enzyme proteins (Brown 1978) and gas chromatographic analysis of terpene composition

(Squillace 1976a). Electrophorectic and gas chromatographic methods offer a number of advantages over quantitative genetic methods to tree improvement: (1) genetic inheritance of biochemical variants can be easily demonstrated; (2) environmental effects are usually small; (3) estimate of genetic variation is directly quantifiable and can be compared between trees, between populations or between species; (4) they are relatively inexpensive and many trees can be screened under uniform and repeatable laboratory environments; and (5) they produce results more rapidly. In addition, expression of variants from enzyme electrophoresis (allozymes) is generally codominant so that homozygous and heterozygous genotypes can be differentiated from each other without the necessity of genetic crosses.

This paper reviews the current status of allozyme and terpene studies in forest trees and discusses the application of biochemical data in tree improvement.

LEVEL OF GENETIC VARIATION

Inherent variation is the raw material for the genetic improvement of forest trees, whether it be by natural (evolutionary) or by artificial (selective breeding) processes. Recent studies have shown that biochemical variation is high for most forest trees. Observed levels of biochemical variation are generally consistent with estimates of genetic variation from provenance/progeny tests. Those species considered quite variable in morphological and physiological traits such as *Picea sitchensis* (Bong.) Carr. (Burley 1966) and *Pinus contorta* Dougl. (Critchfield 1957) also show moderate to high levels of allozyme polymorphisms (Yeh and El-Kassaby 1980; Wheeler and Guries 1982) and considerable variation in terpene spectra (von Rudloff 1978; Forrest 1977). More importantly, morphologically uniform species such as *P. resinosa* Ait. (Wright *et al.* 1972) and *Thuja plicata* Don (Minore 1969) also exhibited extremely low variation in leaf oil terpene composition (von Rudloff and Lapp 1979) and were essentially monomorphic at all or most allozyme loci (Fowler and Morris 1977; Copes 1981).

Parallelism in levels of variation between biochemical and morphological studies suggests that biochemical methods can rapidly evaluate prospects for improving a target species and several of its tree associates. For example, the presence of a large amount of variation in a species would justify the cost of its improvement. On the contrary, the lack of variation in a species would indicate the need for test designs of great precision or the use of interspecific breeding for its improvement. Hence, prior to initiating their improvement, knowledge on relative magnitude of biochemical variation will assist to rank species priority and to determine the strategy to be taken in forest trees. In addition, knowledge of subpopulations containing large amounts of biochemical variation within a target species can be useful for (1) defining areas to be set aside for gene conservation and (2) the selection of trees for use to increase the gene pool in previously selected breeding populations.

DISTRIBUTION OF GENETIC VARIATION AND GEOGRAPHIC PATTERN

Studies to determine how much biochemical variation there is at the level of populations and single-tree progenies within populations (i.e. population structure) have mostly utilized allozymes and are in apparent conflict with morphologically and physiologically based studies of variation. In contrast to reports of tremendous diversity in morphological traits, the overall picture of genetic structure in forest trees emerging from allozyme studies is one of a weakly differentiated series of populations. A small, but significant, proportion of the total allozyme variation ranging between 1,8 percent in inland populations of lodgepole pine *P. contorta* spp. *latifolia* (Dancik and Yeh 1983) to 14,8 percent in *P. monticola* Dougl. (Steinhoff *et al.* 1983), could be attributed to among population differentiation. That the majority of allozyme variation in forest trees is maintained within populations is, perhaps, a reflection of their ecological amplitude, their breeding system, and the lack of effective barriers to gene flow between populations.

Recently, several investigators have shown that multivariate statistical analyses of allozyme data could detect population clusters and/or rich structure of variation associated with geography in forest trees. Contrary to earlier reports on the lack of differentiation in *P. contorta* spp. *latifolia* (Yeh and Layton 1979; Wheeler and Guries 1982; Dancik and Yeh 1983), Yeh *et al.* (1985a) found the extent and pattern of allozyme variation were concordant with results of growth and morphological studies on this conifer (Critchfield 1957; Illingworth 1976). In a study of allozyme variation in *Picea mariana* (Mill.) B.S.P. results from discriminant analysis (Yeh *et al.* 1985b) supported findings from earlier morphological studies of the same trees in insular Newfoundland (Khalil 1975; Khalil and Douglas 1979) that there was considerable genetic differentiation among and within forest sections, and the patterns of differentiation were complex. Guries (1984) also concluded that multilocus analysis of allozyme frequency data could detect non-random groupings of populations which were consistent with groupings based upon provenance/progeny testing data in forest trees. On the basis of these findings, it appears that a large number of small differences at allozyme loci is equivalent to a small number of large differences. Therefore, measures of population differentiation, such as genetic distance (Nei 1972), and *F*-statistics (Wright 1965), might obscure some underlying patterns because all loci are weighted equally.

Clinal patterns of variation among conspecific populations, possibly the expression of underlying genetic processes such as natural selection and past migration patterns, have frequently been found for terpene compositions and allozyme frequencies (e.g. Yeh and O'Malley 1980). The failure to detect clinal patterns of biochemical variation in species such as *Thuja plicata* Don (von Rudloff and Lapp 1979) and *Picea sitchensis* (Bong.) Carr. (Yeh and El-Kassaby 1980) suggests that stochastic factors such as genetic drift and founder effects were important evolutionary forces in the generation and maintenance of the structure of natural populations of such species.

A thorough understanding of a species' variation pattern is prerequisite to its genetic improvement. For example, where population differences in biochemical traits exist, it is possible to delimit, with multivariate procedures, seed or breeding zones within which trees are genetically similar. In addition, comparative patterns of biochemical variation among forest trees can also be used to determine how the selection of parent trees for the base population of a breeding program can maximize the genetic returns from a given investment. Thus, when an observed pattern is such that the level of variation between stands is large relative to the level of variation within stands, it is desirable to emphasize the selection of parent trees at the stand level and devote less effort to selection within stands. Furthermore, where seeds in reforestation programs are pruchased from commercial sources, certification and monitoring of seed sources with biochemical markers would guarantee the genetic quality of plantations.

INTERSPECIFIC VARIATION

Composition of terpenes varied greatly between species (von Rudloff 1975). Similar findings have been found in allozyme data. For example, the average genetic distance between P. contorta spp. latifolia and P. banksiana (Dancik and Yeh 1983) was 20 times greater (0,097) than between populations within species (0,005). Although most of the biochemical studies dealt with taxonomy at the species and generic level, studies of interspecific biochemical variation have been supplementing the use of morphological characteristics for differentiating between hybrids and parental species in the Pinaceae (e.g. Copes and Beckwith 1977). Recently, Yeh and Arnott (1985) have identified key isozymes useful for differentiation between Picea sitchensis, Picea glauca, and their hybrids, and demonstrated the concordance between electrophorectic and morphological data.

While morphological traits have been useful to discern hybrid composition, they are subject to two important limitations. Firstly, morphological characteristics are subject to marked ontogenetic and environmental influences. Thus, to discern hybrid composition with accuracy, hybrid stands must differ substantially in such characteristics from the two parental species. Such a requirement poses problems when low levels of introgression have occurred and/or when hybrid stands vary in their degree of introgression as a result of repeated back-crossing with the parental species. Secondly, genetic composition of the seeds in a stand is determined by both the seed-trees and the pollen pool. Species classification of hybrid seedlots and estimation of their level of introgression on the basis of characteristics of seed-trees can be unreliable when hybrid stands vary in their level of introgression as a result of the spatial and temporal variations in the pollen parent contribution.

The question of species classification for trees and/or seeds collected from stands in which introgressive hybridization between closely related species has occurred is of obvious concern from the perspective of genetic resource management and of ensuring the proper cultural requirements of seedlings in the nursery. For example, incorrect seedlot classification of spruce in British Columbia (B.C.) causes problems in container seedling nursery cultural regimes. If Picea glauca seedlots are not grown under an extended photoperiod at container nurseries in southwest B.C., they form a terminal bud early in the growing season and do not reach the target seedling height (Arnott 1974, 1979). However, if an extended photoperiod is provided for Picea sitchensis, seedlings become unacceptably tall by the end of the growing season (Arnott unpublished data). It is important, therefore, to know which species the introgressive hybrids are more likely to resemble in growth habits; otherwise, significant seedling losses will occur from the crop not meeting or exceeding the specified morphological stock standards set by the B. C. Ministry of Forests.

POLLEN DYNAMICS

Terpenes and allozymes have been useful as genetic markers in studies of pollen dynamics in natural and seed orchard populations. For example, in a nine clone orchard of P. elliottii var. elliottii, analysis of four terpenes showed selfed seedlings were few in number and many seedlings were being produced from matings with contaminant pollen (Squillace 1976b). In allozyme surveys of natural populations, results suggest that forest trees are predominately outcrossers. Published estimates for forest trees ranged from 63 percent outcrossing in Eucalyptus pauciflora Sieb. ex Spreng. (Phillips and Brown 1977) to 100 percent in P. contorta spp. latifolia (Epperson and Allard 1984). Outcrossing rates have been found to differ among populations (Brown et al. 1975; Shaw and Allard 1982), among years within populations (Moran and Brown 1980; Cheliak et al. 1985), and among age classes in geographically proximate populations (Yeh and Meagher unpublished data).

Understanding the mating system of a species is important in the selection of parent trees or provenances, and their subsequent evaluations in replicated tests of open-pollinated progenies. When matings are among near neighbors, in time this will lead to the division of a stand into numerous, small neighborhoods. This information should assist in determining the minimal distances among candidate trees, their number from each stand, and their method of selection (i.e. ocular). Furthermore, adjustments to performance could be made when inbreeding rates are known for families or provenances, since observed differences might reflect primarily the ephemeral differences in inbreeding rates among the families or provenances.

Seed orchards have been established on the assumption that every genotype in the orchard will contribute equally to the next generation (Woessner and Franklin, 1973). It is a common experience, however, that this is not the case (Bergman 1968; Kellison

1971). Differential phenology and production of gametes among genotypes (Jonsson _et al._ 1976) will increase the likelihood of self-pollination and consanguinous mating among relatives in seed orchards. Self-pollination can occur in two ways: within the same tree, and between different ramets of the same clone. Appreciable amounts of self-pollination and inbreeding, and pollen contamination from outside sources in seed orchards, will offset the anticipated gain due to their associated inbreeding depression on yield and performance in vigour and fertility (Franklin 1971; Park and Fowler 1984). For proper choice of seed orchard type, locations, and management practices, therefore, the propensity of a tree species to self-pollinate and to mate among relatives, and the extent of pollen contamination must be known under seedling and clonal orchard settings.

INDIRECT SELECTION

Reports of correlation between biochemical variation and economic traits is scarce for allozymes, but ample for terpenes. El-Kassaby (1982) found enzyme genotype differences at eight loci did not influence quantitative traits to a major extent in _Pseudotsuga menziesii_ (Mirb.) Franco. Mitton _et al._ (1982) found high levels of heterozygosity at enzyme loci were associated with high growth variability in _Populus tremuloides_ Michx. and _P. ponderosa_, and with low variability in _P. contorta_.

Terpene composition was found to relate to resistance against diseases, insects, and animal damage. Wilkinson (1980) found that _P. strobus_ with high a-pinene and low limonene content were generally more resistant to _Pissodes strobi_ Peck than ones with low a-pinene and high limonene. Harris _et al._ (1983) found _Picea sitchensis_ resistant to _Pissodes strobi_ Peck had a characteristic monoterpene spectrum that differed from susceptible trees. Smith (1969) found _P. ponderosa_ Laws. relatively resistant to _Dendroctonus brevicomis_ Lec. beetles had high content of limonene in their stem xylem oleoresin. Radwan and Ellis (1975) found clones of _Pseudotsuga mensiesii_ var. _glauca_ having high total monoterpene per gram of leaf tissue were relatively resistant to deer browsing.

Whether the observed associations between biochemical and economic traits were the results of direct effects of such loci or the coadapted complexes that they mark is problematical. Nevertheless, they do suggest the possibility for indirect selection with biochemical traits in forest trees, especially for resistance to pests and diseases. More research is warranted, in particular, studying multilocus genotypes in full-sib progenies derived from diallel mating designs to reduce the background noise. In addition, the biosynthetic pathways for allozymes and terpenes should be investigated to facilitate biological interpretation of observed associations.

CONCLUSIONS

Biochemical methods can rapidly advance our knowledge pertaining to the amount and pattern of variation in forest trees, provide opportunities for indirect selection, especially for resistance to forest trees pests and diseases, and solve problems encountered in tree improvement. Such problems include identification of trees and seeds of unknown origin, identification of hybrids and determination of their composition, determination of inbreeding and selfing rates in natural and seed orchard populations, and determination of pollen contamination in seed orchards.

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IMPLICATIONS DES SYMBIOSES VÉGÉTABLES DANS L'AMÉLIORATION GÉNÉTIQUE DES ARBRES

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RÉSUMÉ

Après avoir bien mis en évidence les distinctions fondamentales entre les trois principales symbioses racinaires qui se retrouvent chez les arbres forestiers, actinorhizes, ectomycorhizes et endomycorhizes à vésicules et arbuscules, et après avoir sommairement décrit les modalités de leur fonctionnement, l'auteur recommande qu'une grande attention soit accordée à la variabilité quantitative et qualitative des inoculums naturels dans les parcelles expérimentales; dans la plupart des cas il serait avantageux de pratiquer l'inoculation systématique. On énumère ensuite les caractères à rechercher dans l'amélioration génétique des symbiontes utiles ainsi que le potentiel d'amélioration des différents groupes de microorganismes.

SUMMARY

After stressing the fundamental differences between the three main root symbioses of forest trees, actinorhizae, ectomycorrhizae, vesicular arbuscular endomycorrhizae, and after describing the characteristics of their functioning, it is recommended that great care should be given to the qualitative and quantitative variability of natural inocula in the soils of the experimental plots; in most cases it is advantageous to inoculate systematically. Thereafter, an enumeration of desirable characters to breed for in the symbionts is given. A few ideas are given about the potential for improvement in each category of microorganisms.

INTRODUCTION

Dans la nature, la presque totalité des plantes, y inclus les arbres, vivent en symbiose avec des microorganismes du sol qui s'associent étroitement avec leurs racines pour établir des symbioses morphologiquement et physiologiquement intégrées. Contrairement aux concepts des premières heures qui faisaient de ces symbioses des cas d'exception, la totalité des recherches effectuées dans plus de 50 pays au cours des 15 dernières années, concourt à démontrer que les symbioses racinaires constituent un phénomène fondamental et universel chez les plantes vasculaires.

En nature, de même que les plantes ont besoin d'eau et de lumière pour vivre, ainsi ont-elles besoin de leurs symbiontes associés. Si au laboratoire, en serre ou même en parcelles expérimentales les plantes peuvent se développer sans leurs symbiontes, en nature leur nutrition minérale, leur protection contre plusieurs maladies ainsi que leur approvisionnement en eau risquent fort de ne pas se faire adéquatement. La réalité des symbioses végétales s'exprime au niveau écologique.

Le jour où on comprend toute la réalité et la signification de ces propos, on comprend également que l'amélioration des génotypes végétaux doit tenir compte de ces concepts. Ceci est particulièrement vrai en sylviculture.

Si on doit tenir compte des génotypes des microsymbiontes dans l'amélioration des génotypes des arbres, il faut réciproquement tenir compte des génotypes des arbres dans la sélection des microsymbiontes.

Nous soutenons la thèse que la voie la plus prometteuse consiste à sélectionner simultanément les deux membres de la symbiose afin de produire des systèmes symbiotiques à haute performance.

LES TROIS SYMBIOSES LES PLUS FRÉQUENTES ET LES PLUS IMPORTANTES DANS L'AMÉLIORATION GÉNÉTIQUE DES ARBRES

Il existe d'autres symbioses que celles mentionnées ici entre les arbres et les microorganismes e.g. les nodules à Rhizobium, les ectendomycorhizes et les mycorhizes éricoïdes. Cependant dans les régions tempérées du Nord, les trois plus importantes sont celles qui suivent.

La Symbiose Actinorhizienne

Cette symbiose conduit à la formation de nodules bien visibles sur le système racinaire. Le microorganisme est un actinomycète, donc un procaryote; on sait très bien le cultiver axéniquement et il est couramment produit en fermenteur. Il intervient dans la fixation de l'azote atmosphérique et joue un rôle important dans la nutrition minérale azotée des arbres qui le portent, soit dans la succession écologique ou soit dans les plantations mixtes (Gordon et Dawson, 1979).

La Symbiose Ectomycorhizienne

En présence de champignons supérieurs appartenant surtout aux basidiomycètes (eucaryotes), la presque totalité des racines primaires des arbres qui les portent sont morphologiquement et physiologiquement transformées. Sous l'influence du symbionte fongique, l'ensemble de la nutrition minérale de l'arbre est modifiée entre autres en ce qui concerne le phosphore, mais non exclusivement. La résistance à plusieurs maladies fongiques est accrue et la résistance à la sécheresse également. On retrouve ce type de mycorhizes chez les arbres qui se développent naturellement dans les sols avec humus brut; e.g. pin, sapin, épinette, hêtre, bouleau, etc. (Harley et Smith, 1983; Marks et Kozlowski 1973).

La Symbiose Endomycorhizienne à Vésicules et Arbuscules

Formées par des champignons inférieurs (phycomycètes et eucaryotes) les endomycorhizes à vésicules et arbuscules (MVA) ne montrent pas de modification morphologique du système racinaire mais n'en affectent pas moins leur physiologie, de façon similaire aux ectomycorhizes (Powell et Bagyaraj, 1984). On les retrouve sur plus de 90% des espèces de plantes vasculaires. Ce sont surtout les arbres qui viennent normalement dans les humus doux qui vivent cette symbiose; e.g. érable, frêne, orme, thuja, if, etc. Évolutivement, c'est la plus vieille des symbioses racinaires; on la retrouve sur les fossiles de *Rhynia* datant de plus de 350 millions d'années. Ceci suggère que l'ensemble des plantes vasculaires a co-évolué avec les Endogonacées tout au long de leur existence (Malloch *et al.*, 1980).

IMPLICATION MINIMALE DES SYMBIOSES RACINAIRES DANS LA SÉLECTION DES ARBRES

Comme c'est souvent le cas, les connaissances sont plus avancées en ce qui concerne les plantes agricoles et les arbres fruitiers. Aussi il ne faudra pas se surprendre d'y voir de fréquentes références. Une connaissance éclairée de la biologie des symbioses devrait inciter tout améliorateur à en tenir compte du moins de façon minimale sinon intégrale (Fortin et Carlisle, 1984).

Dépendance Symbiotique des Espèces et des Génotypes d'Arbres

Dans les Symbioses Actinorhiziennes

Dans les symbioses actinorhiziennes causées par les *Frankia*, il devrait être évident que les arbres et arbustes qui portent normalement des nodules fixateurs d'azote verront leur comportement écologique entièrement modifié en l'absence de l'endophyte approprié. Ainsi dans un sol appauvri en azote ils deviendront incapables de soutenir la croissance qu'on leur connaît dans de telles circonstances en présence du symbionte. Dans un sol totalement dépourvu d'azote, la plante actinorhizienne devient absolument dépendante de l'endophyte pour tout développement. Dans un sol où on trouve par ailleurs de l'azote, mais en quantité limitée, cette dépendance devient relative jusqu'à devenir nulle lorsque l'azote disponible abonde (Fortin et al., 1984).

Dans une situation de végétation pionnière (e.g. recul du glacier, déchets de mines, bancs d'emprunt), la dépendance des plantes actinorhiziennes est absolue. Au cours de la succession primaire, à mesure que les produits de la fixation symbiotique s'accumulent, la dépendance actinorhizienne devient relative. Dans les plantations mixtes, l'association avec un arbre efficace à utiliser l'azote maintient l'azote du sol en quantité sub-optimale; ainsi la plante actinorhizienne, et indirectement l'espèce associée, demeurent relativement dépendantes de la présence et du fonctionnement de l'endophyte. Dans un sol abondamment fertilisé, cette dépendance devient nulle.

Dans les Symbioses Mycorhiziennes

La notion de dépendance mycorhizienne est, semble-t-il, plus difficile à percevoir. Peut-être en est-il ainsi parce que cette dépendance, contrairement à celle des actinorhizes, ne peut pas être absolue. En absence de tout phosphore dans un sol, il n'est pas possible, même en présence d'endophytes mycorhiziens compatibles, d'obtenir quelque croissance que ce soit; la situation est différente de celle rencontrée avec les actinorhizes.

C'est dire qu'avec les mycorhizes, la dépendance de la plante-hôte demeure toujours relative, sauf en présence d'un excès de phosphore soluble où elle devient nulle. On aura compris que pour que le système mycorhizien fonctionne, il faut que le sol contienne une bonne réserve de phosphore sous forme peu soluble; c'est le cas dans la grande majorité des sols naturels, surtout ceux qui sont utilisés pour la sylviculture.

La dépendance mycorhizienne au champ (DMRCP), telle que définie par Plenchette et al. (1983), est fonction de l'espèce de plante vasculaire et de la teneur en phosphore soluble (plus précisément sa vitesse de mise en circuit) du sol. Pour un sol donné ayant une teneur donnée en P soluble, il est possible de classer les espèces de plantes selon un ordre de dépendance qui peut aller de 100 à 0%.

Pour le même sol et en utilisant les mêmes plantes, si la teneur en P soluble est augmentée, l'ordre relatif de dépendance des espèces restera le même mais pour chacune, le degré de dépendance deviendra moindre; les plantes qui s'étaient avérées les moins dépendantes dans le premier cas pourraient même devenir indépendantes. Par contre si la teneur en P soluble pouvait être abaissée, on verrait le degré de dépendance de toutes les espèces augmenter, jusqu'au point où des espèces totalement indépendantes dans le premier scénario montreraient une dépendance.

Les expériences effectuées avec les *Citrus* démontrent très bien que cette dépendance relative ne se retrouve pas seulement entre espèces mais aussi entre cultivars. Chez le pommier, Granger *et al.* (1983), ont montré que deux cultivars distincts se sont montrés différemment dépendants vis-à-vis les MVA. Cependant le cultivar le moins dépendant supporte un développement beaucoup plus important du système racinaire aux dépens des produits de la photosynthèse.

Il nous apparaît donc clairement que la formation et le fonctionnement des mycorhizes ne sont pas seulement liés aux espèces de plantes vasculaires mais aussi aux génotypes à l'intérieur de l'espèce.

Spécificité Hôte-Endophyte

La question de spécificité hôte-symbionte n'est pas aussi simple qu'on l'aimerait. Dire qu'il y a une certaine spécificité laisse perplexe, mais c'est pourtant le cas.

Dans le cas des *Frankia*, ceux-ci se répartissent en groupes d'inoculation où un ensemble de souches forment des actinorhizes avec un groupe donné de plantes hôtes, mais non avec un deuxième groupe et vice versa. Ainsi les souches normalement associées au genre *Alnus* ne nodulent pas les espèces du genre *Eleagnus* et réciproquement (Normand, 1985).

Chez les ectomycorhizes, certains genres d'arbres acceptent comme symbiontes une multitude de genres et d'espèces fongiques. Ainsi les pins (Trappe, 1962), les mélèzes (Samson, 1985) et le peuplier faux-tremble (Godbout et Fortin, 1985), au laboratoire du moins, acceptent un très grand nombre de champignons alors que l'*Alnus crispa* (Godbout et Fortin, 1983) ne s'associe qu'à de très rares espèces de champignons. Entre ces deux extrêmes, on retrouve les sapins et les épinettes.

Chez les champignons ectomycorhiziens eux-mêmes certaines espèces peuvent s'associer, toujours au laboratoire, à de nombreux genres et espèces d'arbres. C'est le cas avec le *Pisolithus tinctorius*, le *Laccaria bicolor*, le *Thelephora terrestris*, le *Paxillus involutus* (Trappe, 1962). D'autre part, dans les essais réalisés jusqu'ici, l'*Alpova diplophloeus* n'a jamais formé d'ectomycorhizes à l'extérieur du genre *Alnus*.

Chez les endomycorhizes à vésicules et arbuscules, le nombre d'espèces fongiques impliquées semble beaucoup plus restreint (quelques centaines sur la planète) et apparemment ces mêmes champignons peuvent s'associer à plus de 100 000 espèces de plantes vasculaires. Par exemple, la quelque centaine d'espèces de plantes vasculaires réunies dans l'érablière laurentienne de la vallée du Saint-Laurent partagent une ou peut-être deux douzaines d'espèces fongiques endomycorhiziennes.

Dans le genre *Alnus*, l'*Alnus crispa* peut former à la fois des actinorhizes et des ectomycorhizes alors que l'*Alnus glutinosa* peut former, en plus des actinorhizes, des endomycorhizes MVA et aussi des ectomycorhizes. Plusieurs espèces de peuplier peuvent former indifféremment des encomycorhizes MVA ou des ectomycorhizes; une symbiose à quatre partenaires peut donc exister.

Contrairement aux organismes pathogènes contre lesquels les arbres cherchent à se protéger, on n'observe pas chez les mycorhizes de spécificité aussi étroite; il semble que chaque espèce de plante-hôte est le plus souvent ouverte et capable de s'associer à un grand nombre de micro-symbiontes. Ce sont éventuellement les conditions du milieu et, en particulier, les conditions édaphiques qui déterminent les associations à privilégier.

L'efficacité des Microsymbiontes Associés

Quels que soient les microsymbiontes impliqués, il est toujours possible de voir l'association se former mais demeurer relativement ou totalement inefficace.

Variabilité de l'Abondance Naturelle des Propagules des Divers Symbiontes dans les Sols

Après avoir pris conscience du caractère fondamental et de l'universalité des symbioses racinaires chez les plantes vasculaires et notamment chez les arbres, il serait erroné de conclure que les propagules des divers symbiontes, actinorhiziens, ectomycorhiziens et endomycorhiziens, abondent nécessairement et en tout temps dans le milieu naturel, dans les parcelles utilisées pour la sélection génétique ou sur les sites de plantation. De plus si elles abondent sur un site, il peut arriver qu'elles ne soient ni compatibles ni efficaces.

Différents événements sont susceptibles de perturber les populations naturelles des microsymbiontes et c'est le cas notamment avec le feu, avec l'utilisation des biocides et des fertilisants, avec les pratiques culturales telles que le labour qui "dilue" les propagules et les rotations où la présence de certaines plantes est défavorable à la multiplication d'une catégorie d'endophyte donnée.

Ainsi les sols des pépinières et des parcelles expérimentales où s'effectuent les sélections génétiques, bien qu'ils apparaissent physiquement et chimiquement (fertilisation maximale) homogènes, sont le plus souvent biologiquement hétérogènes. Parmi les organismes du sol, ce sont les microsymbiontes qui exercent les effets les plus rapides et les plus marqués sur le développement des plants. Ainsi dans les sites où ces propagules abondent, la symbiose s'installe dès les premiers instants, permettant ainsi un départ rapide de la croissance; par ailleurs, dans les microsites où les propagules sont absentes, un délai de plusieurs semaines peut avoir lieu dans la formation de la symbiose, ce qui peut se traduire par une différence considérable dans les taux de croissance. Dans de telles conditions, les variations "phénotypiques" observées entre les plants risquent de traduire plus la variation dans l'abondance des propagules que dans les génotypes des plantes elles-mêmes; bien sûr, avec des répétitions et des fertilisations massives peut-on obvier en partie à cette lacune, mais dans quelle mesure et à quel coût?

D'autre part, est-il souhaitable d'effectuer les sélections en présence de concentrations élevées de fertilisants? Dans la nature c'est en association avec leurs symbiontes que les arbres assureront leur nutrition minérale.

Quant à la résistance aux maladies fongiques, jusqu'à quel point les méthodes actuelles ne conduisent-elles pas à la sélection de génotypes résistant à la fois aux champignons mycorhiziens et conséquemment moins aptes à s'associer efficacement avec les symbiontes tout en devenant plus en plus exigeants en fertilisants?

Importance de l'Inoculation dans la Sélection des Génotypes d'Arbres

À la lumière de ce qui précède, nous concluons qu'il serait nécessaire de s'assurer que les propagules des symbiontes compatibles avec l'hôte sont présentes en quantités optimales dans les substrats utilisés pour la sélection des génotypes d'arbre. Cet objectif peut être atteint en partie par la détermination des nombres les plus probables de propagules et par le piégage *in situ*, mais le moyen le plus sûr demeure l'inoculation avec des souches compatibles et efficaces.

Lorsque les génotypes sont propagés *in vitro*, l'inoculation peut être pratiquée dans le tout jeune âge de la plantule avec un minimum d'inoculum, ce qui réduit considérablement les coûts. En parcelles expérimentales, l'opération est un peu plus onéreuse, mais reste tout à fait praticable, qu'il s'agisse des actinorhizes, des ectomycorhizes ou des endomycorhizes MVA.

CARACTÈRES À RECHERCHER DANS L'AMÉLIORATION GÉNÉTIQUE DES SYSTÈMES SYMBIOTIQUES IMPLIQUANT DES ARBRES

Caractères Relevant des Hôtes

Dans le développement d'un système symbiotique hôte-microbionte performant, l'arbre devrait avoir pleine capacité 1° à former l'association, 2° à la nourrir en substances énergétiques et 3° à profiter des produits de l'association (e.g. azote fixé, phosphore accumulé, etc.). Tremblay (1985) a obtenu en collaboration et propagé par culture *in vitro*, un clone d'*Alnus crispa* incapable à toute fin pratique de former des nodules après inoculation avec les *Frankia*.

On recherchera donc chez l'hôte des génotypes permettant pleinement la formation des symbioses ainsi que leur fonctionnement optimum.

Caractères Relevant des Endophytes Racinaires

La sélection des génotypes de microsymbiontes performants semble laisser plus de latitude.

Dans le cas des Frankia, on recherchera ceux

- qui permettent la formation de masses nodulaires importantes
- qui permettent une nodulation rapide
- qui permettent une fixation maximale de l'azote
- qui ont un moindre coût photosynthétique
- qui forment des nodules à durée de vie prolongée
- etc.

Dans le cas des ectomycorhizes et des endomycorhizes, les génotypes à privilégier sont ceux qui permettent

- la transformation de la plus grande proportion des racines réceptives en ectomycorhizes
- une grande efficacité à recueillir les éléments nutritifs peu mobiles, e.g. le phosphore
- une grande efficacité à éloigner les organismes pathogènes tels que champignons et nématodes
- un coût énergétique minimum
- une plus grande efficacité à détoxifier les métaux lourds
- une adaptation aux conditions édaphiques, pH, température, etc.

POTENTIEL D'AMÉLIORATION GÉNÉTIQUE DES FRANKIA

Comme les Frankia appartiennent aux procaryotes, ils se prêtent volontiers aux manipulations génétiques. En effet, la préparation des protoplastes, l'identification et la transformation des plasmides peuvent s'effectuer à peu près comme chez les bactéries. La génétique des actinomycètes ayant reçu une grande attention dans le secteur médical, il est relativement facile de transposer et d'adapter les techniques existantes. Ainsi, non seulement on peut obtenir facilement des mutants par méthodes classiques mais il est d'ores et déjà possible d'effectuer des transformations par génie génétique; ces méthodes permettent d'améliorer les propriétés déjà connues des Frankia et laissent entrevoir l'introduction de nouvelles propriétés chez ces microsymbiontes (Normand, 1985).

La symbiose des Frankia avec un nombre limité d'espèces ligneuses laisse plus d'espoir d'être élargie à de nouvelles espèces que n'importe lequel autre microsymbionte, y compris les Rhizobium. Sachant que les Frankia, contrairement aux Rhizobium, sont capables de conduire seuls toutes les opérations conduisant à la fixation de l'azote et sachant également que cette symbiose est la plus récente dans le monde végétal (35 millions d'années), il apparaît probable que le nombre de gènes impliqués chez chacun des partenaires soit réduit et, par conséquent, la faisabilité de transporter cette symbiose chez d'autres espèces apparaît comme très possible. Le bouleau fixateur d'azote apparaît donc comme plus immédiatement réalisable que le blé fixateur d'azote.

POTENTIEL D'AMÉLIORATION GÉNÉTIQUE DES CHAMPIGNONS ECTOMYCORHIZIENS

Les études sur la génétique et la biologie cellulaire des champignons ectomycorhiziens sont à toute fin pratique inexistantes. Bien qu'on imagine facilement que ces champignons se comportent à peu près comme les autres basidiomycètes, il n'existe actuellement aucune publication faisant état du croisement de mycéliums ectomycorhiziens haploïdes suivi de la fécondation et de la méïose et ayant conduit avec succès à une deuxième génération de spores. Quelques travaux ont montré l'existence d'une certaine variabilité tant sur des souches haploïdes que diploïdes mais à cause de la difficulté considérable à obtenir jusqu'ici la fructification (caryogamie et méïose) de la majorité des espèces, on peut dire que la génétique classique de ces mycosymbiontes reste entièrement à faire.

Quant à la biologie moléculaire, les premières préparations de protoplastes régénérées (Kropp *et al.*, 1985) viennent à peine de voir le jour et la recherche des plasmides s'amorce à peine. Encore là, tout est à faire.

POTENTIEL D'AMÉLIORATION GÉNÉTIQUE DES CHAMPIGNONS ENDOMYCORHIZIENS À VÉSICULES ET ARBUSCULES

La particularité de ces importants mycosymbiontes, c'est que dans l'état actuel des connaissances, leur culture axénique reste impossible. Le seul mode de propagation accessible consiste à cultiver le champignon sur plante-hôte et à utiliser les spores produites ou encore des racines portant le mycelium (Plenchette, 1983). On comprendra que ceci limite sérieusement, pour le moment du moins, les études sur la biologie moléculaire et la génétique de ces champignons.

Compte tenu de l'universalité et des effets de ces mycosymbiontes sur les plantes et les arbres, il existe un besoin énorme de recherches de base sur ce phénomène biologique aussi ancien que les végétaux vasculaires et profondément inscrit dans les génomes des associés (Trappe et Molina, 1985).

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PROGRAMMES DE MICROPROPAGATION RÉALISÉS À LA STATION D'AMÉLIORATION DES ARBRES FORESTIERS DE L'I.N.R.A.

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RÉSUMÉ

Différents programmes de micropropagation conduits à la Station d'Amélioration des arbres forestiers de l'I.N.R.A. sont présentés. Celui du merisier (Prunus avium L.) est le plus développé. Après un rappel sur la technique de micropropagation mise au point et employée, est présentée l'évolution de ce programme au niveau du nombre de clones sélectionnés (201), multipliés (146), installés en tests clonaux (88) et commercialisés (28 en 1984, 100 000 plants). Le programme noyer (Juglans sp.) fait l'objet d'un développement important: l'utilisation d'embryons ou de rejets de souches a permis des progrès conséquents. L'introduction en culture (à l'exception des contaminations) comme la multiplication sont maîtrisées, des approfondissements sont nécessaires pour l'enracinement. D'autres programmes: Mélèzes (Larix sp.), Peupliers (Populus sp.) et érable (Acer platanoides) font l'objet de premières recherches.

ABSTRACT

Various micropropagation programs set up at the forest tree improvement station of the national agricultural research institute of France (INRA) are discussed. The wild cherry (Prunus avium L.) program is the most advanced. After a review of the micropropagation technique developed and used, the evolution of the program is presented in regards to the number of clones selected (201), multiplied (146), installed in clonal tests (88), and commercialized (28 in 1984, 100 000 plants). The walnut (Juglans sp.) program is under important development: the use of embryos or of sprouts has led to useful progress. Introduction into microculture (except for contaminations) as well as multiplication are under control but deeper studies are needed on rooting. Other programs on larches (Larix sp.), poplars (Populus sp.) and maple (Acer platanoides) are getting underway.

Le groupe d'Amélioration des Arbres Forestiers de l'Institut National de la Recherche Agronomique (Bordeaux et Orléans) a pour objectifs principaux d'accroître en volume et en qualité la production de bois d'oeuvre. Une bonne conduite et une efficacité maximale des programmes d'amélioration nécessitent des recherches physiologiques: induction florale, résistance à l'hydromorphie, technologie des graines, incompatibilité de croisements, juvénilité et vieillissement, multiplication végétative,...

Cette dernière est particulièrement importante aussi bien pour des objectifs appliqués (vergers à graines, production massale de clones,...) que scientifiques (interaction génotype-milieu, résistance aux maladies,...). À l'Unité de Physiologie d'Orléans, différentes techniques sont expérimentées: bouturage, greffage, drageonnage et, depuis 1979, la micropropagation *in vitro*.

La micropropagation *in vitro* est actuellement étudiée ou en projet d'étude pour 5 espèces importantes pour la qualité de leur bois, les besoins en reboisement, mais aussi en raison des difficultés rencontrées pour les propager à l'aide d'autres techniques.

Prunus avium L. (Merisier): Les besoins en matériel de qualité pour le reboisement sont urgents.

Juglans sp. (Noyer): En particulier les hybrides interspécifiques (*J. nigra* L. x *J. regia* L.) très difficiles à multiplier par bouturage.

Larix x *eurolepis* A. Henry. (Mélèze hybride): obtenu en quantité très limitée par croisement contrôlé après induction florale.

Acer pseudoplatanus L. (forme ondée) et *Populus* sp. (Peupliers grisards: *P. alba* x *P. tremula* L. et *P. alba* L. x *P. tremuloïdes* Michx.): ils font seulement l'objet d'études préliminaires.

LE MERISIER

Produisant un bois de haute qualité pour l'ébénisterie, disséminé dans la majorité des forêts françaises, il est surexploité. Devant la pénurie de matériel de qualité pour le reboisement, un programme de sauvegarde et de multiplication des meilleurs phénotypes fut entrepris fin 1977. Les principales étapes de ce programme sont:

- Sélection en forêt,
- Multiplication végétative,
- Tests clonaux et constitution de variétés polyclonales régionales,
- Création de nouveaux clones par croisement.

Méthode de multiplication:

Le bouturage des arbres adultes donne des résultats très faibles. Si des drageons apparus sur des grosses racines peuvent s'enraciner, l'obtention de ceux-ci est aléatoire et seulement 25 pour cent des clones peuvent être mobilisés. Seule la micropropagation *in vitro* a permis de résoudre le problème.

La technique mise au point a déjà été publiée (Riffaud et Cornu 1981; Cornu et Chaix 1982). Elle est maintenant couramment employée tant au niveau recherche qu'au niveau commercial.

Mise en culture: Les contaminations posent le problème majeur pour l'introduction du matériel collecté en forêt. L'utilisation de chlorure mercurique ($HgCl_2$ à 0,1 pour cent: matériel herbacé ou 0,5 pour cent: matériel ligneux) permet l'obtention de plus de 80 pour cent de clones sains.

Dans 60 pour cent des cas, le matériel devient réactif (les bourgeons introduits *in vitro* reprennent leur croissance) après 1 à 2 transferts. Néanmoins, certains clones nécessitent des repiquages fréquents durant parfois un an, avant d'atteindre un taux de multiplication régulier.

Multiplication: Selon les clones, deux milieux minéraux sont essentiellement utilisés: les macroéléments de Murashige et Skoog (M.S.) légèrement modifiés (Riffaud et Cornu 1981) ou ceux du P.W.M. (Lloyd et McCown 1980). Généralement, les clones sont cultivés peu de temps sur ce dernier milieu.

Les meilleurs taux de multiplication (5 à 8 toutes les cinq semaines) sont obtenus pour 85 pour cent des clones avec l'équilibre en régulateurs de croissance suivant: acide indolbutyrique (AIB) 1 mg/l, acide gibberellique (AG) 0,1 mg/l, N benzylaminopurine (BAP) 1 mg/l. Certains clones, sensibles à la transformation hyperhydrique, nécessitent une dose plus faible en régulateurs de croissance (AIB 0,5 mg/l, BAP 0,1 mg/l).

Une phase d'élongation est nécessaire avant l'enracinement. Elle est effectuée sur le même milieu avec AG (10 mg/l) uniquement.

Enracinement: Cette phase demeure la phase la plus délicate et la plus variable. Elle nécessite:

- Une réduction de la concentration du milieu en macro-éléments (au 1/5°).
- Une réduction du niveau lumineux (200 lux au lieu de 2000 lux) et de la température (19°C au lieu de 22-25°C).
- L'adjonction de 0,5 à 5 mg/l d'AIB selon les clones.

Dans ces conditions, 40 pour cent des clones s'enracinent à plus de 90 pour cent, et autant entre 60 et 90 pour cent. Dans certains cas d'enracinement moyen, il est possible d'obtenir l'apparition des racines après transfert en serre. Celles-ci, commencées *in vitro*, sont probablement inhibées par la présence de l'auxine dans le milieu.

Actuellement, pour accroître la réaction des clones réfractaires, les recherches sont conduites sur l'action des facteurs lumière et température qui se sont déjà révélés importants.

Déroulement du programme:

Sélection: Les sélections ont débuté en 1978 dans le Nord de la France puis progressé vers le Sud (tableau 1, Figure 1).

Tableau 1. Situation au printemps 1985 du nombre de clones sélectionnés et multipliés *in vitro*

Clones sélectionnés et récoltés	Clones multipliés *in vitro*			
	Introduits	Perdus	En cours d'étude	Transférés en pépinière
201	185	39	30	116
Pourcentages	100	21	16	63

Sur les 39 clones perdus, 21 l'ont été par contamination et 18 par absence de reprise de réactivité.

Tests clonaux: Les conditions écologiques françaises sont très variables et nécessitent l'installation de nombreux tests clonaux pour permettre de juger le comportement des clones (tableau 2). Établis dès 1982, ils permettront la création de variétés polyclonales régionales à partir des meilleurs clones.

La micropropagation *in vitro* conduit à la production de matériel physiologiquement juvénile. Celui-ci se bouture alors aisément sous forme herbacée (sur 93 clones expérimentés en 1983, 62 se sont enracinés à 69 pour cent et 22 à plus de 90 pour cent). Néanmoins, la réalisation d'un parc de pieds-mères constitue un frein à une diffusion rapide de cette technique.

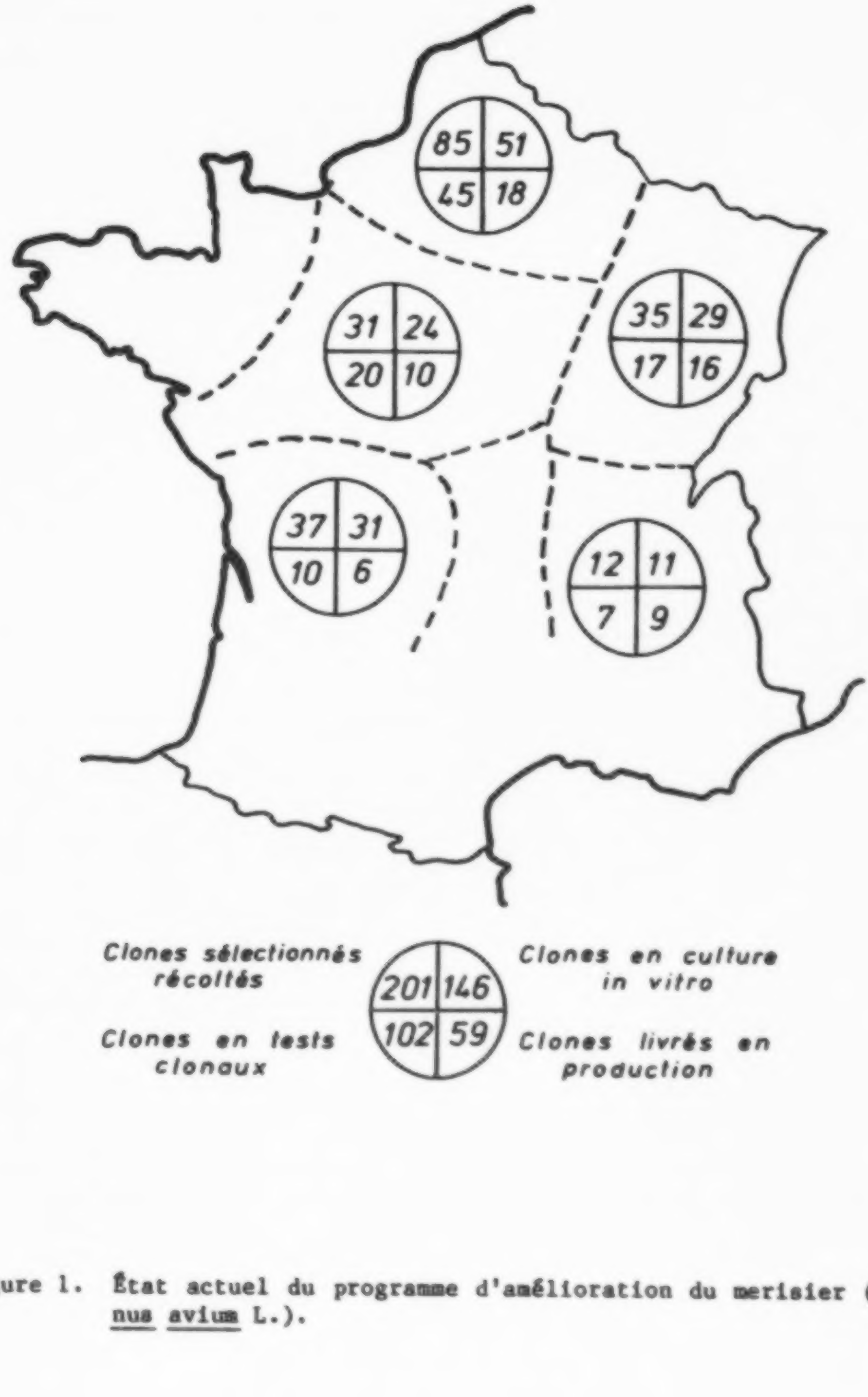
85
51
45
18
31
24
20
10
35
29
17
16
37
31
10
6
12
11
7
9
Clones sélectionnés récoltés
Clones en culture in vitro
201
146
102
59
Clones en tests clonaux
Clones livrés en production

Figure 1. État actuel du programme d'amélioration du merisier (Prunus avium L.).

Tableau 2. Principaux dispositifs installés dans les grandes régions naturelles françaises à l'aide de plants issus de micropropagation. À titre de comparaison 3 dispositifs comportent des plants bouturés

Année	Région	Superficie	Nombre de clones		Remarques
			Total	Locaux	
1982	Nord	1 ha	17	9	9 clones communs
	Est	1 ha	19	8	
	Centre	1 ha	17	10	
1983	Centre	0,7 ha	20	9	60% de boutures
1984	Est (Champagne)	2 ha	66	11	30% de boutures
1985	Nord (Picardie)	1 ha	34	11	34 clones communs
	Nord (Normandie)	1 ha	34	18	
	Sud-Ouest (Poitou)	1 ha	34	3	
	Sud-Ouest (Limousin)	1 ha	34	3	
	Est (France-Comté)	1 ha	34	12	
	Nord (Picardie)	0,5 ha	16	9	70% de boutures
Total		11,2 ha	88		

Production commerciale: Les besoins actuels sont importants (700 000 plants par an en moyenne). Bien que les résultats des tests clonaux ne soient pas encore disponibles, dès 1981 un premier lot de clones a été livré au secteur commercial en conseillant leur utilisation dans leur seule région d'origine.

La production actuelle des clones sélectionnés pour le reboisement est uniquement assurée par micropropagation *in vitro*. Quatre laboratoires privés disposent de clones mais un seul produit la majorité des plants (tableau 3, figure 1).

Tableau 3. Évolution de la production commerciale pour le reboisement de plants de Merisier produits par micropropagation

Année	Nombre de clones	Nombre de plants	Utilisateurs principaux
1982	5	20 000	1 pépinière
1983	10	35 000	2 pépinières
1984	28	100 000	5 pépinières, I.D.F.*
1985 (projet)	15	100 000	

* I.D.F. = Institut pour le Développement Forestier.

Le prix de vente d'un plant issu de micropropagation et préparé au repiquage en pépinière est voisin de 4 francs. Après un an de croissance en pépinière et pour une hauteur comprise entre 75 cm et 1 m, il vaut 12 francs. Le prix d'un semis d'origine indéterminée est de 5 francs pour la même dimension.

LE NOYER

Pour cette espèce, seule la multiplication végétative et particulièrement la micropropagation *in vitro* peuvent permettre de résoudre le grave problème de déficit en matériel performant pour le reboisement.

Le programme a débuté en 1981 (Jay-Allemand 1982) et a pour objectifs principaux:

- Multiplier rapidement, par culture d'embryons, les descendances obtenues par croisement dirigé.
- Multiplier les meilleurs clones sélectionnés après rejuvénilisation.

Matériel juvénile: De très bonnes familles hybrides interspécifiques (ex.: NG23xRA, INRA, Arboriculture, Bordeaux) sont disponibles ou en cours de création. Mais le nombre de noix produites est réduit. Un programme de multiplication de ces noix par culture d'embryons a été récemment entrepris.

Les embryons cultivés sur le milieu de Knop sans régulateur de croissance, présentent une forte réaction au saccharose. Les faibles concentrations (20 à 40 g/l) favorisent le développement de l'épicotyle et des feuilles, les fortes concentrations (80 g/l) favorisant celui des racines.

Repiqué sur le milieu de Miller (1967) en présence de BAP (1-2 mg/l) les épicotyles sans racine développent en moyenne 7 bourgeons. Après une élongation lente, ceux-ci constituent le point de départ d'une nouvelle multiplication.

Matériel âgé: Deux points ont longtemps constitué un frein au développement de la technique:

Les contaminations: le matériel est extrêmement contaminé, en particulier par des bactéries qui pourraient être pour certaines endogènes. Néanmoins un maximum d'explants indemnes de bactéries peut être obtenu à l'aide de matériel herbacé en phase de croissance très active, environ 3 semaines après le débourrement.

Perte de réactivité avec l'âge: le matériel lignifié libère très rapidement des composés phénoliques dans le milieu de culture et meurt. Seul le matériel herbacé peut conduire à l'établissement de cultures.

Le niveau de réactivité décroît avec l'âge du pied-mère. Néanmoins, certaines techniques (greffage, taille sévère et surtout recépage) permettent une réacquisition importante de cette réactivité *in vitro* (tableau 4). C'est cette dernière technique qui nous a permis, en 1984, d'introduire, de multiplier et d'enraciner avec succès un arbre âgé de 34 ans à partir de rejets de souche.

Tableau 4. Évolution de la réactivité d'explants en culture selon l'âge ou le traitement des pieds-mères

Âge ou traitement du pied-mère	Réactivité *in vitro* en pour cent
Arbre de 30 ans	5
Plant de 3 ans	26
Plant de 3 ans recépé	60
Semis de 3 mois	77

Parmi tous les milieux expérimentés, c'est le milieu DKW de Driver et Kuniyuki (1984) qui donne les meilleurs résultats en particulier au niveau qualitatif. En présence d'AIB (0,001 mg/l) et de BAP (1,5 mg/l) une moyenne de 4 à 5 bourgeons peut être obtenue par explant. La kinétine et l'isopenténnumeric adénine n'améliorent pas les résultats. Une grande variabilité clonale est observée entre les clones hybrides, les meilleurs appartenant au groupe des Paradox (*J. hindsii* Jeps. x *J. regia* L.).

Une forte dominance apicale se manifeste même en présence d'une dose élevée de cytokinine et un seul bourgeon s'allonge. L'AG et le charbon actif sont inhibiteurs, en particulier ce dernier (tableau 5), peut-être par piégeage de la cytokinine.

Tableau 5. Action du charbon actif sur deux clones en phase de multiplication (2 fois 2 semaines de culture)

	Sans charbon		Charbon 500 mg/l	
	108	134	108	134
Nombre moyen de bourgeons	4	5	1	1,3
Allongement moyen de la plus grande pousse en mm	16	23	8	12

Des repiquages fréquents avec élimination du cal sont nécessaires. En effet, dans tous les cas, nous observons une production importante de cals qui au bout de 2 semaines inhibe le développement ultérieur de l'explant.

Selon cette technique, 15 clones sur 18 introduits ont été établis en cultures en 1984. Une multiplication abondante de deux de ces clones a permis d'obtenir les premiers enracinements sur le milieu de DKW dilué au 1/5 en présence d'AIB (1-5 mg/l). Les racines produites sont néanmoins grosses et accompagnées d'une formation abondante de cals réduisant les chances de reprise au transfert. Actuellement, quelques plants poursuivent avec succès leur croissance en serre.

LE MÉLÈZE

Si la multiplication d'arbres adultes sélectionnés fait partie des objectifs de ce programme, l'effort principal est mis sur la multiplication "en vrac" (Bulk propagation) sans identification clonale des meilleures familles hybrides obtenues par croisements dirigés.

Parmi différents milieux expérimentés, le milieu N30K (Margara 1977) et le milieu M.S. (dilué de moitié) donnent les meilleurs résultats avec les deux types de matériel.

Matériel adulte: Les brachyblastes, prélevés avant le débourrement (février à avril), conduisent à la production de 6 à 12 bourgeons axillaires en présence d'auxine (AIB 0,1 mg/l) et de cytokinine (BAP 1,5 mg/l). Néanmoins, nous n'avons pas pu obtenir l'élongation de ces bourgeons.

Matériel juvénile: Les explants sont prélevés sur des plantules de 30 jours. Ils sont constitués de l'hypocotyle et des cotylédons coupés à moitié. À l'aide de BAP (2 mg/l) 2 à 6 bourgeons cotylédonnaires peuvent être obtenus. Deux mois au moins sont nécessaires pour obtenir une élongation de 2 cm. Celle-ci n'est pas accélérée par l'utilisation de charbon actif ou d'AG. Sur un milieu dilué au 1/5^{e} en présence d'AIB (1 mg/l) l'enracinement atteint 60 pour cent. Après transfert, les plants ont une croissance active et orthotrope.

Les travaux engagés qui avaient été interrompus seront poursuivis afin d'augmenter la production des bourgeons.

AUTRES PROGRAMMES

Si les travaux sur l'Érable ont été peu développés, des résultats très satisfaisants sont obtenus avec les Peupliers grisards. Sur le milieu M.S. (dilué de moitié) additionné de BAP (0,1 mg/l) les 4 clones expérimentés ont été multipliés avec succès. Des taux de multiplication supérieurs à 10 pour cent et un enracinement voisin de 100 pour cent rendent cette technique compétitive pour des clones traditionnellement multipliés par boutures de racines (drageonnage).

De nombreux autres programmes liés à la micropropagation *in vitro* sont conduits en coopération avec d'autres équipes.

- Caractérisation biochimique (composés phénoliques) de la juvénilité, de la maturité et de la rejuvénilisation chez le Noyer.
- Mycorrhization *in vitro* du merisier pour accroître la reprise et la croissance des jeunes plants.
- Études bactériologiques: assainissement de cultures contaminées, étude de la résistance au Crown-gall (*Agrobacterium tumefaciens*) chez le Peuplier et le Merisier.
- Cultures de protoplastes de Merisier.

CONCLUSION

Le programme de micropropagation des arbres forestiers a démarré depuis peu d'années. Il a pourtant répondu avec succès aux aspirations tant des améliorateurs que des praticiens puisque déjà près de 15 pour cent des plants de Merisier installés en forêt sont issus de culture *in vitro*. Dans les prochaines années, ce développement sera privilégié chaque fois que la multiplication clonale sera adoptée comme objectif d'amélioration ou de reboisement.

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TRADITIONAL FOREST GENETICS vs BIOTECHNOLOGICAL AND PHYSIOLOGICAL APPROACHES

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ABSTRACT

After 60 years, tree improvement is now an accepted forest management strategy. Still, tree improvement is costly and time consuming. There are a number of proven techniques, including isozyme analysis and terpene identification, that have assisted in describing genetic differences among tree species. The new biotechnology, including genetic engineering, somaclonal screening, parasexual hybridization and advanced tissue culture, offers forest geneticists techniques for the direct transfer of genetic information at any stage of tree development. The new techniques in conjunction with traditional methods can provide the means for greater efficiency in tree improvement programs.

Additional keywords: Biotechnology, genetic engineering, somaclonal screening, parasexual hybridization, cell fusion, tissue culture.

RÉSUMÉ

Après 60 ans, l'amélioration des arbres est maintenant une stratégie d'aménagement forestier reconnue. Elle est cependant coûteuse en argent et en temps. Nombre de techniques éprouvées dont l'analyse des isoenzymes et l'identification des terpènes ont permis de décrire les différences génétiques entre les espèces d'arbres. La nouvelle biotechnologie, comprenant le génie génétique, le tamisage somaclonal, l'hybridation parasexuelle et la culture de tissus, offre aux généticiens forestiers des techniques de transfert direct de l'information génétique à toutes les étapes du développement de l'arbre. Conjuguées aux méthodes traditionnelles, ces nouvelles techniques peuvent apporter aux programmes d'amélioration des arbres des moyens leur permettant d'être plus efficaces.

Mots-clés additionnels: biotechnologie, génie génétique, tamisage somaclonal, hybridation parasexuelle, fusion cellulaire, culture de tissus.

INTRODUCTION

In the last 60 years, forest genetics and tree improvement have come a long way. Research and development activities by a few pioneers has now lead to an accepted strategy of forest management. An indication that our science has come of age is the fact that most major forestry organizations, either public or private, now have a tree improvement component as part of their forest management program. Another sign that we have matured is the fact that forest genetics activities are no longer immune from major budget cuts, and that the costs of doing tree improvement are being challenged. In many ways we have followed the path of agriculture with an overriding concern for productivity and less of a concern for efficiency. In the next few years we can expect more challenges from our budget officers and serious competition from other forest management programs for the limited funding.

Another sign of our maturity appears to be our reluctance to accept and to use new methods and technology. We have become comfortable with our proven and tried skills. To a degree this may be a slight overstatement, but some of the newer techniques available to forest geneticists are not as readily accepted as in the past. In our youth, as a new forestry discipline, we sought out technologies that advanced and accelerated our science. Considerable progress has been made because we were innovative in our approaches to understanding the genetics of forest trees.

The basic goals of forest genetics have not essentially changed in the last 60 years. We are still interested in faster growth, improved adaptability, improved wood quality and greater disease and insect resistance. We achieved many of the goals for a number of tree species over time by devoting our research energies to testing and screening tree species, to identifying superior populations, by carrying out interspecific, and intraspecific breeding programs, by initiating multitrait breeding programs and now by gene manipulation. To accomplish the above goals we have employed and developed an array of research techniques that were directed toward improved seed production, including flowering stimulation, pollen management and insect and disease control. We further sharpen our skills in cloning by directing research on vegetative propagation, grafting and budding, and tissue culture. To reduce cost and increase efficiency, as well as quality, we spent considerable research resources in the area of seed collection, handling and storage, improved nursery practices and developed newer greenhouse procedures including containerization. To accelerate actual genetic improvement we extended and even initiated new areas of population genetics.

CURRENT RESEARCH TECHNIQUES

Still, the improvement of forest trees is difficult. Their large size and long life present serious constraints. Their delayed reproductive systems present a major challenge. To understand differences between species and populations we have sought the means of describing the material we have worked with. Formerly, we employed terpene analysis for selected conifers such as the pines. Such methods assisted in the description of species and to a degree permitted us to separate populations. The original techniques were slow and time consuming. With the advent of gas chromatography, rapid and more accurate analysis could be obtained. Even small biochemical differences could be identified. Distinct populations could be identified chemically. At about the same time, in the late 1950's, we employed another technique of the protein biochemist, the isozyme method. This method was originally developed to purify proteins, while the original gas chromatographic procedures were developed to separate and identify amino acids. It should be noted that this technique and the information provided was not universally accepted by our field.

Isozyme methodology, in addition to providing a useful method for describing our plant material, makes it possible for us to better understand our systems at the molecular level. Originally isozyme techniques provided us with a powerful tool to further separate species, populations and eventually individual trees. Currently the technique is being used to establish linkage maps for selected conifers, and we can expect the techniques to be applied to selected hardwoods.

We have now added an additional tool to our trade. It goes under many names made popular by the general press but for our discussion I shall use the term the new biotechnology. Under this collective term I shall include the areas of genetic engineering, somaclonal techniques, cell fusion, and tissue culture. Much has been written about this new technology and the question we need to explore is its role in forestry. For this discusion I will limit my comments to forest genetics.

NEW BIOTECHNOLOGY

There seem to be some uncertainties in accepting this new technology and understanding the role it should play with our traditional systems. This uncertainty is reflected by Dr. Libby's comments at your 1983 symposium on clonal forestry when he noted: "promise of faster entry of new clones to production forestry through molecular biology appears to me to be a fantasy and perhaps a dangerous one" (Libby 1983). He is in my opinion wrong. Of course clonal forestry has a major role to play and is playing it in getting improved material into production. Clonal forestry has been with us for many years and as efficiency of clonal propagation improves we will see it more widely used; and that is as it should be. The new biotechnology is, of course, a new "science". The field is only approximately 12 years old and its application to forestry less than 5 years old. The promise of the new biotechnology will be its ability to address our age-old problem of bypassing the natural sexual process and the long life of trees. The new science offers us the opportunity to direct genetic change at the molecular level by selective manipulation. It is, of course, a high risk technology, and one that has to be entered into with care and a high degree of caution. If we are successful, we will be able to develop a tree improvement timetable not unlike the agriculturist. We will be able to quickly and effectively move individual genes within and between tree species. We will be able to address, in time, such recurring concerns as disease resistance, selection for given soil conditions such as alkalinity or salinity, the science of nitrogen fixation in forest trees, herbicide resistance, and gene mapping.

The tools we are currently developing include new methodology of gene-splicing (recombinant DNA), parasexual hybridization (cell fusion), and somaclonal selection.

Genetic Engineering

In gene-splicing, or recombinant DNA, we are interested in the identification, isolation and transfer of desirable genes. For forest geneticists this remains a big order since few useful genes are known and none are mapped. This is an area of serious research. Still a few simply inherited traits are known, including genes dealing with disease resistance. Here, biotechnology will help, in that, with the use of DNA-probes combined with high resolution isozyme mapping, we will be able for the first time to seriously generate a gene library for forest trees. Lest you think that such approaches are academic in nature, such research is already underway with several tree species. An array of enzymes (restriction enzymes) are now available for the isolation of DNA fragments. Techniques are also now available to multiply or clone these DNA fragments. Within the last several years systems have been developed for transferring known fragments of genetic information from one organism to a different one, including forest trees. The most common carrier is the Ti plasmid from the common crown gall bacterium (*Agrobacterium tumefaciens*). In a few cases the genetic information has been transferred directly by microinjection. The point is that in less than 4 years we now have a new set of research tools.

Cell Fusion (Parasexual Hybridization)

Cell fusion research has been underway for sometime. Such methodology may have limited use with trees but should not be overlooked. A major constraint has been our lack of ability to regenerate whole plants from single cells except in a few tree species. This remains a barrier for certain types of gene transformation experiments as well. At this time I am aware of only two tree species that have been regenerated from a single cell, i.e., *Populus* sp., *Alnus* sp. Still the method is a useful tool for both nuclear and cytoplasmic inheritance.

Somaclonal Screening

Finally, research is underway using somaclonal techniques. Here individual cells are exposed to an array of environments. Such a method may yet prove, in some cases, to be more efficient in screening for herbicide tolerance, and disease resistance than conventional techniques. Again we are dealing most frequently with single cells, although new research suggests this need not be the only type of plant material that needs to be tested. Current studies suggest an array of tissues might well be used. Coupled with traditional field testing procedures, we have a much more powerful tool.

Tissue Culture

Considerable research has now been focused on tissue culture as a means for clonal forestry. Improved methods of regenerating single cells to whole plants are vital to advancing selected biotechnology methods. With more attention on forest trees we already see considerable progress. This remains a major area of research that can benefit many different areas of forest genetics.

CONCLUSION

Earlier, I disagreed with the assessment that the contribution from biotechnology is many years off. I noted a number of serious constraints to the new science, yet I still implied that the new science has a more immediate role to play. Within the last 6 months there is good evidence that the Aro-A-gene has been inserted into the tissue of *Populus*. The Aro-A-gene has been demonstrated to confer tolerance to the herbicide glyphosate in higher plants. The gene was isolated from bacteria by Calgene, a California biotechnology company. The point here is that genes from other organisms can be used by trees and that individual genes can be transferred. The concept will obviously need some additional testing, but there appears to be no need for long-term testing. The herbicide tolerance can be tested in young trees. There has already been some success in regenerating cells of *Populus*, and we certainly know how to clone *Populus*. Furthermore, recent studies of the last year have demonstrated that genetic transformation need not take place only in single cells but has taken place with an array of structures including leaf explants, cell suspensions and even cotyledons. Should these systems work equally as well with trees then the research and development in transformation and somaclonal techniques will be accelerated. Lest you feel that this is remote it should be noted that an array of single and linked genes have been transferred successfully in over 30 higher plants and the genes are active.

Some 60 years ago we embarked on a vigorous program of forest genetics and tree improvement. This program was considered somewhat naive and high risk by the agriculturist of that day as well as many in forestry. The new biotechnology program of today is considered by some in forestry to be ill-advised. We have set sail in an ocean of uncertainty but the ultimate promise of success far outweighs the risks. If forest genetics is to be competitive and if we are to successfully address the issue of efficiency, we will need an array of new techniques to strengthen our current efforts. The research needs are many, yet in a relatively few years progress has been considerable. I see no reason why the new sciences will not become a vital part of our tool chest. The new techniques will not displace traditional methods, but supplement them.

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CONFÉRENCIERS VOLONTAIRES

VOLUNTARY PAPERS

IN VITRO PROPAGATION OF SOME CONIFERS BY COTYLEDON AND EPICOTYL CULTURE

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ABSTRACT

Plantlets were regenerated from segments of cotyledons, hypocotyls, and the epicotyl with cotyledons in black spruce (*Picea mariana* (Mill.) B.S.P.), white spruce (*P. glauca* (Moench) Voss), jack pine (*Pinus banksiana* Lamb.), and eastern white pine (*P. strobus* L.).

Explants were incubated on modified GD medium supplemented with 10 mg/l benzylaminopurine (BAP) and 0.2 mg/l naphthaleneacetic acid (NAA) for 40 days. The proliferated explants with primordia of adventitious shoots were then transferred to 1/2 strength of GD medium supplemented with 1% activated charcoal, 1 mg/l BAP, and 0.2 mg/l NAA, for another 30 days for shoot induction. Further shoot development and elongation was induced by culturing the explants on 1/2 strength of GD medium. An average of 22 adventitious shoots were produced from each segment of cotyledon or epicotyl in six months in black spruce, 15 from epicotyl in jack pine, and 8 from epicotyl in white spruce and eastern white pine. When the adventitious shoots reached 1 cm in height, they were rooted using pulse treatment or in 1/2 strength of GD medium containing either NAA, perlite or vermiculite. Roots developed in 30 days and the plantlets were then established in soil.

RÉSUMÉ

Des plantules ont été produites à partir de segments de cotylédons, d'hypocotyles et d'épicotyle avec cotylidons, d'épinette noire (*Picea mariana* (Mill.) B.S.P.), d'épinette blanche (*P. glauca* (Moench) Voss), de pin gris (*Pinus banksiana* Lamb.) et de pin blanc (*P. strobus* L.).

Les explants ont été incubés sur un substrat GD modifié additionné de 10 mg/l de benzylaminopurine (BAP) et de 0.2 mg/l d'acide naphthalénacétique (NAA), pendant 40 jours. Les explants ayant proliféré en primordia de tigelles adventives ont été ensuite transférés sur un substrat GD de concentration 1/2, additionné de 1% de charbon de bois, 1 mg/l de BAP et 0.2 mg/l de NAA, pendant 30

jours, pour induire la pousse. Le développement des tigelles et l'élongation additionnelle ont été induits par la culture des explants sur un substrat GD de concentration 1/2. En moyenne, chaque segment de cotylédon ou d'épicotyle d'épinette noire a produit en six mois 22 tigelles adventives; pour l'épicotyle de pin gris, ce nombre est de 15, pour l'épicotyle de l'épinette blanche et du pin blanc, de 8. Quand les tigelles atteignaient 1 cm de hauteur, on les enracinait par traitement de choc ou sur un substrat GD demi-concentré contenant de l'NAA, de la perlite ou de la vermiculite. Les racines se développèrent en 30 jours et les plantules furent alors mises en terre.

INTRODUCTION

Vegetative propagation is preferred in clonal forestry because genetic make-up of desirable genotypes of forest trees is well maintained. Traditionally, vegetative propagation has been carried out by rooting of cuttings or grafting. In vitro propagation of embryonic or seedling explants is a new technique that could be applied for vegetative propagation. During the last few years, significant progress has been made in achieving organogenesis from explants of forest trees. Plantlets have been successfully regenerated from explants of germinants in Norway spruce (P. abies) (von Arnold 1982), black spruce (P. mariana) (Rumary and Thorpe 1984), white spruce (P. glauca) (Rumary and Thorpe 1984), loblolly pine (P. taeda) (Brown and Sommer 1977, Mehra-Palta et al. 1978), radiata pine (P. radiata) (Aitkin et al. 1981), lodgepole pine (P. contorta) (Patel and Thorpe 1984), and eastern white pine (P. strobus) (Minocha 1980).

The present investigation was aimed at inducing adventitious shoots on a large scale from excised cotyledons, epicotyl and hypocotyl, and rooting the shoots in black spruce, white spruce, jack pine and eastern white pine.

MATERIALS AND METHODS

Open-pollinated seeds of black spruce, white spruce, jack pine and eastern white pine were used in these experiments. Seeds of black spruce, white spruce and jack pine were sterilized for 20 minutes in 10% Javex bleach solution with two drops of Tween 20 per 100 ml and washed four times with sterilized distilled water. Eastern white pine seeds were stratified by embedding them in wet sand for 40 days in a refrigerator at 5°C and sterilized as for other species. All the seeds were germinated on 0.6% agar in petri dishes in an incubator at 25°C during the day and 15°C at night with a 16-hour photoperiod. One week after germination, the whole seedling was resterilized for 8 minutes with 10% Javex bleach solution and washed in sterilized distilled water. Segments of cotyledon and hypocotyl (about 1 cm in length) and epicotyl with intact cotyledons were excised and incubated on 3 kinds of culture media in petri dishes under the same culture conditions as for seed germination (Figs. 1 and 2).

The three different culture media were 1) Murashige and Skoog (MS) (1962), 2) Schenk and Hildebrandt (SH) (1972), and 3) Gresshoff and Doy (GD) (1972). The plantlets were regenerated in the following four steps.

Step 1: Organogenesis and induction of adventitious shoots. Explants were incubated on each of the three media, supplemented with 10 mg/l benzylaminopurine (BAP) and 0.2 mg/l naphthaleneacetic acid (NAA), 3% sucrose and 0.6% agar for about 40 days.

Step 2: Development of adventitious shoots. The proliferated explants with shoot primordia were transferred to the 1/2 strength of each of the basic media supplemented with 1 mg/l BAP, 0.2 mg/l NAA, 1% activated charcoal, 2% sucrose and 0.3% agar for 30 days.

Step 3: Elongation of the shoots. The explants with shoots were transferred to the 1/2 strength of basic media supplemented with 2% sucrose, and 0.3% agar. These media were used for all subsequent transfers.

Step 4: Rooting of the shoots. Adventitious shoots of about 1 cm in height were excised and rooted in the 1/2 strength of each of the media supplemented with 0.25% agar, and either 1) 0.2 mg/l NAA (shoots were pulse-treated for 5 days and then transferred to medium without growth regulator), 2) 0.02 mg/l NAA, 3) 0.1 mg/l indolebutyric acid (IBA), 4) 0.1 mg/l phloroglucinol, 5) perlite, or 6) vermiculite. The agar medium was carefully washed from the plantlets which were then planted in promix soil (peat moss, vermiculite and perlite = 3/1/1) in pots. The potted plants were kept in a mist chamber for 2 to 4 weeks before transferring to the greenhouse.

RESULTS

Development of adventitious shoots

Explants began to proliferate after 8 to 10 days of incubation. In about 30 days, most proliferated cotyledon segments developed shoot primordia along the length of the cotyledons (Fig. 3), and few hypocotyl segments produced the primordia. In the epicotyls with intact cotyledons, the primordia were mainly regenerated from the axil of cotyledons (Fig. 4). The percentage of primordial formation in the 3 types of explants in black and white spruce, and jack and eastern white pine on GD medium are shown in Table 1. Cotyledon was the best material for shoot induction in black spruce while epicotyl was best for jack pine and eastern white pine. In white spruce, epicotyl and hypocotyl was better than cotyledon.

The results of the culture of three types of explants in jack pine and black spruce incubated on three kinds of media indicated that SH medium was the best for jack pine and cotyledon was the most responsive material in shoot primordia induction (90%). In black spruce, GD medium was the best and cotyledon (90%) was more responsive to the induction than epicotyl (80%) and hypocotyl (15%). MS medium was inferior to both SH and GD medium in primordial induction in both species.

After another 30 days of incubation of the media with charcoal followed by 20 days of incubation on the media without charcoal or plant growth regulator, the primordia developed and elongated to adventitious shoots (Figs. 5 and 6). The shoots were excised for rooting in two harvests with a 10-day interval. A maximum of three harvests were carried out for each explant. The number of adventitious shoots per explant in black spruce, white spruce and jack pine is shown in Table 2. Black spruce averaged 22 shoots per explant while jack pine had an average of 15 shoots per explant. White spruce and eastern white pine were poor in shoot production with an average of 8 per explant in each species.

Rooting of adventitious shoots

Of the six treatments, the best results were obtained by pulse-treatment for black spruce, in which an average rooting percentage of 53 was achieved in 30 days (Figs. 7, 8 and 9). In the medium containing either perlite or vermiculite, the shoots rooted equally well with an average of 33%. In jack pine, the medium containing 0.02 mg/l NAA was better than that used for the pulse treatment and 50% of the shoots produced roots, in contrast to a rooting percentage of 33 in pulse treatment and 13 in the medium containing either perlite or vermiculite. In white spruce and eastern white pine, poor rooting percentage resulted in all the treatments, and the pulse treatment was the best giving 10% rooting. No adventitious shoots in any of the 4 species studied were rooted in the medium with either IBA or phloroglucinol.

DISCUSSION

The capability of explants to form adventitious shoots depends on the type of explant, the treatment of the explant donor, the genotype of the species, and the species (Aitkin et al. 1981, von Arnold 1982). Patel and Thorpe (1984) found that intact embryos excised from seeds were superior to cotyledon and hypocotyl segments in shoot production in lodgepole pine while Rumary and Thorpe (1984) favored the use of epicotyl with cotyledons in black and white spruce. The present study agreed with the reports that the epicotyls with cotyledons were more responsive to the induction in white spruce, jack pine, and eastern white pine. However, cotyledon segments appeared to be better than epicotyl and hypocotyl in black spruce.

Advances in rooting of adventitious shoots have been made in recent years. Rancillac et al. (1982) successfully rooted adventitious shoots in maritime pine (P. pinaster) using pulse treatment. Rumary and Thorpe (1984) obtained the best results in black and white

Table 1. Shoot forming capacity of different types of explants on GD medium in black spruce, white spruce, jack pine and eastern white pine

Species		No. of explants incubated	No. of explants with shoots	% of explants with shoots
Black spruce	cotyledons	200	160	80
	epicotyl	80	55	69
	hypocotyl	120	18	15
White spruce	cotyledons	78	29	37
	epicotyl	56	27	48
	hypocotyl	58	27	47
Jack pine	cotyledons	100	48	48
	epicotyl	40	28	70
	hypocotyl	60	20	33
Eastern white pine	cotyledons	45	10	22
	epicotyl	40	24	60
	hypocotyl	40	3	8

Table 2. The number of adventitious shoots produced from each explant in black spruce, white spruce and jack pine

No. of shoots per explant in the range of:	Black spruce		White spruce		Jack pine	
	No. of explants	Avg. No. of shoots	No. of explants	Avg. No. of shoots	No. of explants	Avg. No. of shoots
0 - 10	33	6	30	3	35	5
11 - 20	18	14	25	14	20	12
21 - 30	12	24	-	-	10	22
31 - 40	6	35	-	-	5	32
41 - 50	12	42	-	-	6	41
50	9	56	-	-	2	52
Average		22		8		15

spruce by dipping the shoots in a rooting powder. In the present study, pulse treatment was found to be the most effective for root induction in black spruce, white spruce, and eastern white pine. Adventitious shoots of jack pine rooted well in a medium containing a low concentration of NAA.

The production potential of plantlets from each germinant varies from species to species. Based on an average of 22 adventitious shoots per explant in black spruce, a germinant producing an average of 10 explants could regenerate about 220 shoots and these would give a production potential of about 120 plantlets (53% rooting). In jack pine, the production potential of plantlets would be about 75 per germinant. Aitkin et al. (1980) reported production of 150 plantlets per seed in radiata pine while Patel and Thorpe (1984) produced 17 plantlets per explant with a maximum of 37 in lodgepole pine. Von Arnold (1982) obtained 15 plantlets per explant in Norway spruce and Minocha (1976) produced 6 plantlets from each embryo in eastern white pine.

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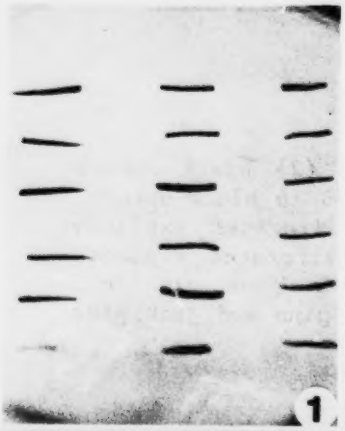
ILLUSTRATIONS (Overleaf)

(1) segments of jack pine cotyledons, (2) black spruce epicotyl with cotyledons, (3) proliferated cotyledons in black spruce, (4) proliferated epicotyls in jack pine, (5) proliferated explants with adventitious shoots in black spruce, (6) proliferated explants with adventitious shoots in jack pine, (7) white pine plantlet, (8) black spruce plantlets, (9) black spruce, white pine and jack pine plantlet (left to right).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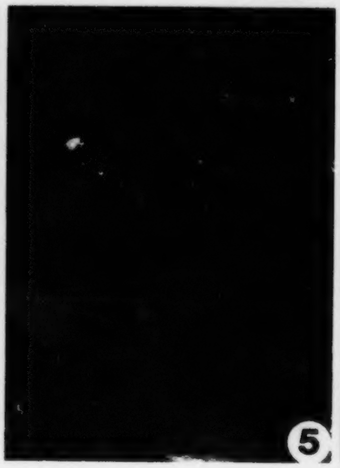
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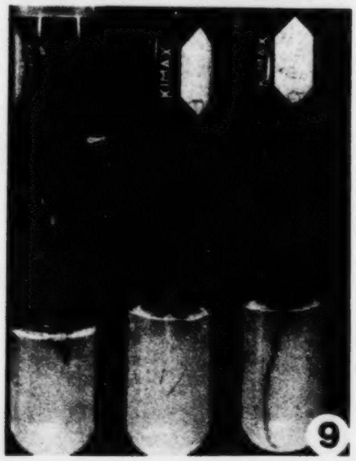
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INITIATION OF CALLUS AND SHOOT PRODUCTION IN PRUNUS SEROTINA

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ABSTRACT

Black Cherry (Prunus serotina), one of the most valuable hardwoods in the northeastern United States, has been propagated from lateral bud explants and successfully transferred to soil following establishment, multiplication and rooting in vitro. However, to increase the rate of mass regeneration of genetically improved clones at an economically acceptable cost, regeneration from cell cultures is being explored.

Using axenic stem segments from micropropagated plantlets as explants, we have found that Gamborg's B-5 media supplemented with 30 g/l of sucrose, 3 mg/l IBA (Indole butyric acid), and 1 mg/l BAP (Benzylaminopurine), produces the healthiest (green to yellow) and largest quantities of callus. Callus has been produced on 13 clones and requires subculturing every two to four weeks depending on the individual clone's growth rate. Some clones have been subcultured up to eight times.

Out of four clones tested to date, one has been induced to produce shoots from callus when transferred to a state II micropropagation media (Murashige and Skoog media supplemented with 20 g/l sucrose, 0.01 mg/l IBA, 0.75 BAP; Tricoli, Maynard, Drew, Forest Science, 31:201-208, 1985). These shoots were induced to form roots on a stage III media and have been transferred to a soilless potting mix.

Experiments are currently being established to develop a shoot regenerating media using both juvenile and mature tissue of roots, leaves, and shoots as explant material. Calli from these explants are being tested for shoot regenerating capabilities.

RÉSUMÉ

Le cerisier noir (Prunus serotina), l'un des feuillus les plus précieux dans le nord-est des États-Unis, a été multiplié à partir d'explants de bourgeons latéraux et transféré avec succès dans du

sol après établissement, multiplication et enracinement in vitro. Cependant, pour augmenter le rythme de multiplication en masse de clones génétiquement améliorés à un coût économiquement acceptable, on est en train d'explorer la régénération par culture de tissus.

En utilisant comme explants des segments axéniques de tiges de plantules micromultipliées, nous avons découvert que la formule de substrat B-5 de Gamborg, renforcée de 30 g/l de sucrose, de 3 mg/l d'IBA (acide indole butyrique) et de 1 mg/l de BAP (benzylaminopurine), produit les plus saines (vertes à jaunes) et les plus grandes quantités de cals. Du cal a été produit sur 13 clones et demande à être repiqué toutes les deux à quatre semaines selon le rythme de croissance du clone impliqué. Certains clones ont été repiqués jusqu'à huit fois.

Des quatre clones testés jusqu'à ce jour, un a été amené à produire des tigelles à partir du cal lorsqu'on l'a transféré sur un substrat de micromultiplication de deuxième étape (substrat de Murashige et Skoog renforcé de 20 g/l de sucrose, de 0,01 mg/l d'IBA, de 0,75 mg/l de BAP; Tricoli, Maynard et Drew, Forest Science, 31:201-208, 1985). Ces tigelles ont été amenées à s'enraciner sur un substrat de troisième étape et ont été transférées sur un mélange pour contenant sans sol.

Des expériences sont en cours et visent à mettre au point un substrat pour la régénération des tigelles en utilisant comme explants des tissus aussi bien juvéniles qu'adultes issus de racines, de feuilles et de tigelles. Les cals de ces explants sont testés pour leur capacité de régénérer des tigelles.

MICROPROPAGATION OF AMERICAN ELM

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ABSTRACT

Cloning of American elm (Ulmus americana L.) was carried out by culturing the hypocotyl and epicotyl with 2 cotyledons from germinants. After 3 months of culture, MS medium supplemented with 1 mg/l of BAP and 0.02 mg/l of NAA was found best for induction of adventitious shoots from a callus regenerated from an epicotyl and it averaged 2.8 shoots per callus. Some of the germinants were planted in 1/2 MS basal medium which contained 1/2 strength of the macronutrients in MS medium. The terminal shoot was excised at the internode above the lowest pairs of leaves and planted in 1/2 MS basal medium. The terminal shoot rooted in 10 days and at least 10 plantlets were regenerated from a single donor plant in 3 months.

Keywords: Ulmus americana, Tissue Culture, in vitro Culture, Cloning, Dutch Elm Disease

RÉSUMÉ

On a pratiqué le clonage de l'orme d'Amérique (Ulmus americana L.) en cultivant l'hypocotyle et l'épicotyle avec deux cotylédons de germinants. Après trois mois de culture, le substrat MS additionné de 1 mg/l de BAP et 0.02 mg/l de NAA s'est révélé le meilleur pour induire des tigelles adventives sur un cal multiplié à partir de l'épicotyle, avec en moyenne 2.8 tigelles par cal. Certains germinants ont été plantés sur un substrat 1/2 MS qui contenait la moitié de la concentration de macronutriments du substrat MS. La pousse terminale a été excisée à l'entrenoeud au-dessus des paires inférieures de feuilles et plantée dans le substrat 1/2 MS. Cette pousse terminale s'est enracinée en 10 jours et au moins 10 plantules ont été régénérées en 3 mois à partir d'une seule plante-mère.

Mots-clés: Ulmus americana, culture des tissus, culture in vitro, clonage, maladie hollandaise de l'orme.

INTRODUCTION

The devastating Dutch elm disease (*Ceratocystis ulmi*) has killed millions of elm (*Ulmus* spp.) in North America and Europe. The disease is transmitted from tree to tree by bark beetles carrying the fungus. In the past few years, the fungal infection has abated due to an integrated program of pest management through biological controls (Strobel and Lanier 1981). On the other hand, investigators are trying to find a solution to the devastating problem through *in vitro* culture. Ulrich *et al.* (1984) produced plantlets from callus which has been preserved through cryopreservation. This cryopreservation could be used to preserve the genotypes that may be irretrievably lost due to insects and diseases. Using cell suspension cultures, Durzan and Lopushanski (1975) regenerated plantlets from callus which was derived from the cells. Lin *et al.* (1981) cultured explants from twigs and young leaves of a 10-year-old Japanese elm (*U. propinqua* or *U. japonica*) tree and obtained some plantlets from the regenerated calli. Attempts were also made to culture immature embryos (Anagnostakis 1977) and to induce haploids through anther culture in American elm (Redenbaugh *et al.* 1981).

Aseptic clonal material is valuable in facilitating studies of the infection mechanism and pathogenicity of *C. ulmi* and identifying plant genes for disease resistance breeding. The present study was designed to clone each donor plant by culturing the explants from aseptic germinants of American elm.

MATERIALS AND METHODS

Open-pollinated seeds were collected from several American elm trees. Seed coats were removed and the naked seeds were sterilized with 10% Javex bleach solution plus two drops of Tween 20 for 15 minutes. They were rinsed 3 times with sterilized distilled water before being placed on a 0.6% agar medium for germination. Seeds germinated within a week. As soon as the green cotyledons were open, each epicotyl with two cotyledons was separated from the hypocotyl and incubated on Murashige and Skoog (MS) medium (1962) supplemented with various combinations and concentrations of benzylaminopurine (BAP) and naphthaleneacetic acid (NAA), along with a 1 cm segment of the proximal end of the hypocotyl from the same donor plant, in a Petri dish (60 x 20 mm). Sixteen kinds of media were made from four concentrations each of BAP (0.1, 1, 2, and 3 mg/l) and NAA (0.02, 0.2, 1, and 2 mg/l). Twenty cultures were made for each medium. The media were adjusted to pH 5.8 and then autoclaved at 1 kg/cm^2 for 15 minutes. The cultures were kept at 25°C during the day and 15°C at night under a 16-hour photoperiod with a PPFD at 15 E/ m^2/sec in an incubator.

Some of the germinants were transplanted, one to a jar containing a modified MS medium which had 1/2 strength of the macronutrients in MS medium. Twenty jars were kept in a growth room having culture conditions similar to the incubator. When a germinant grew to a plant with three pairs of large leaves in addition to a pair of cotyledons, the terminal was excised at the internode above the lowest pair of leaves and planted into the modified medium with or without 0.02 mg/l of NAA for rooting. A larger plant with more than 3 pairs of leaves could produce a terminal shoot and an explant with a pair of leaves for rooting. Plantlets were regenerated from lateral shoots of a donor plant or an explant, and from subsequently rooted cuttings.

RESULTS

After a month of culture of the epicotyl with two cotyledons, the brownish green calli produced a few adventitious shoots and some shoot primordia in all the sixteen media while in the media with high concentrations of NAA, the calli regenerated not only shoots but also roots (Table 1). After three months of culture, MS medium supplemented with 0.02 mg/l of NAA, and 1 or 4 mg/l of BAP gave the best results producing an average of about 3 shoots per callus, ranging from 1 to 8 shoots per callus. However, shoot growth and development was better on the medium with 0.02 mg/l of NAA and 1 mg/l of BAP than that with 0.02 mg/l of NAA and 4 mg/l of BAP. Calli incubated on MS medium supplemented with a high concentration of NAA (1 or 2 mg/l) and low BAP (0.1 mg/l) produced more than 5 adventitious roots per callus and as many as 24 roots were formed on a single callus.

Table 1. Average number of shoots or roots per callus regenerated from epicotyl with two cotyledons which were cultured on MS medium supplemented with various combinations and concentrations of BAP and NAA

		BAP (mg/l)							
		0.1	1	2	4	0.1	1	2	4
		Average No. of shoots per callus				Average No. of roots per callus			
NAA (mg/l)	0.02	0.7	2.8	1.7	2.9	0.1	0	0	0
	0.2	0.9	1.8	0.9	0.5	0.6	0.1	0	0
	1.0	0.8	0.8	0.7	0.4	6.7	0.6	0.3	0
	2.0	0.6	0.5	0.6	0.4	5.0	0.3	0	0

No adventitious shoots were regenerated from the greenish brown calli originated from hypocotyl segments. However, two calli incubated on MS medium supplemented with a low concentration of BAP and high NAA, produced 1 or 2 roots per callus after 3 months of culture.

Shoots, either terminal or lateral, and cuttings with a pair of leaves, rooted equally well in both the modified MS medium with and without 0.02 mg/l of NAA. In some cases, only leaves of a terminal shoot were partially embedded in the medium, and a root was produced from the cut end and grew into the medium. In 3 months of culture, over 10 plantlets were regenerated from each donor.

DISCUSSION

This study demonstrated that American elm could be rapidly cloned in a short period of time. Because the resulting plantlets are aseptic and clonal, this would provide material to facilitate replicated studies on fungal infection, screening for disease resistance, and progeny testing.

Multiplication of adventitious shoots from calli regenerated from epicotyl with cotyledons in American elm appeared to be best when it was incubated on MS medium supplemented with 1 mg/l of BAP and 0.02 mg/l of NAA (Table 1). A similar combination and concentration of BAP and NAA was used in successful regeneration of adventitious shoots from calli induced from twig and young leaf explants in Japanese elm (Lin et al. 1981). Ulrich et al. (1984) produced plantlets from cryopreserved calli originated from the hypocotyl of American elm by using woody plant medium with coconut milk and high concentrations of BAP (4 mg/l) and NAA (1 mg/l). Durzan and Lopushanski (1975) omitted m-inositol, vitamins, and plant growth regulators from the medium in order to produce adventitious shoots from calli regenerated from suspension culture cells of hypocotyl origin.

High concentrations of BAP and low concentrations of NAA tended to induce regeneration of adventitious shoots, while concentrations high in NAA and low in BAP seemed to induce formation of adventitious roots (Table 1). Durzan and Lopushanski (1975) also obtained roots when cells were plated on a medium high in auxin (2.5 mg/l of NAA) and low in cytokinin (1 mg/l of kinetin). Since American elm calli are sensitive to the ratio of auxin and cytokinin in shoot or root regeneration, they make a good specimen to study gene regulation in organogenesis.

In response to cultural treatments, epicotyl with cotyledons appeared to be superior to hypocotyl for regeneration of adventitious shoots. Most calli regenerated from epicotyls produced shoots while none of the calli from the hypocotyl segments produced any shoots in 3 months of culture. However, cells from suspension culture established from friable calli regenerated from hypocotyl segments of American elm, were induced to form shoot primordia in 50 days of culture (Durzan and Lopushanski 1975).

A genotype is best maintained by *in vitro* rooting of cuttings and this would provide clonal material for genetical and physiological studies. On the other hand, plantlet production through callusing and shoot regeneration can result in somaclonal variation.

Therefore, plantlets regenerated from cuttings would provide better material for an accurate assessment of within and among clonal variation in a disease resistance study or genetic testing.

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DEVELOPMENT OF RESISTANCE IN POPULUS TO SEPTORIA MUSIVA UTILIZING SOMACLONAL VARIATION

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ABSTRACT

The identification and isolation of variant cells, cell aggregates, calli, or adventitious shoots in aseptic cultures of several important agronomic crops has subsequently resulted in regenerated plants which express resistance to a variety of pathogens. If the type of variation described above (i.e. variation observed in plants obtained from aseptic culture systems, and given the term "somaclonal variation") can be identified and isolated in aseptic cultures of forest tree species, it would be possible to consider for the first time the improvement of specific characteristics in a period of months rather than decades. The goal of this cooperative research project is to develop a model system for identifying somaclonal variation in aseptic cultures of selected hybrid *Populus* clones and regenerate plants that express an increased resistance to the fungal pathogen *Septoria musiva*. This fungus is a stem and foliar pathogen that limits the usefulness of many hybrid poplar clones in the north central and northeastern United States. Somaclonal variation offers the possibility of minimizing this threat by obtaining resistance to *S. musiva* in these otherwise valuable genotypes.

Keywords: aseptic culture, tree improvement, resistance screening.

RÉSUMÉ

L'identification et l'isolation de cellules variantes, agrégats de cellules, cals ou tigelles adventives dans des cultures aseptiques de plusieurs plantes agricoles importantes ont par la suite donné des plants multipliés qui manifestent une résistance à toute une gamme de pathogènes. Si ce type de variation (c'est-à-dire celle que l'on observe pour les plantes obtenues dans des systèmes de culture aseptique et qu'on appelle "variation somaclonale") pouvait être identifié et isolé par la culture aseptique d'essences forestières, il serait alors possible de considérer pour la première fois l'amélioration de caractères spécifiques sur une période de quelques mois plutôt que de quelques décennies. Le but de ce projet coopératif de recherche est de mettre au point un système pour identifier la variation somaclonale dans des cultures aseptiques de clones choisis de Populus hybrides et pour multiplier les plants qui démontrent une résistance accrue au champignon pathogène Septoria musiva. Ce champignon est un pathogène de la tige et des feuilles qui réduit l'utilité de nombreux clones de peupliers hybrides dans le nord et le nord-est des États-Unis. La variation somaclonale offre la possibilité de réduire cette menace en obtenant une résistance à S. musiva chez ces génotypes qui autrement seraient utiles.

Mots-clés: culture aseptique, amélioration des arbres, sélection pour la résistance.

INTRODUCTION

Hybrid poplars are currently receiving considerable attention as sources of fiber and energy. However, in the north central and northeastern United States, potential biomass yields from intensively managed hybrid poplar plantations are primarily limited by the stem and foliar pathogen Septoria musiva. Before hybrid poplars can be economically utilized by the forest industry, genotypes resistant to S. musiva must be available.

The North Central Forest Experiment Station's (NCFES) Biotechnology Program is part of the U.S. Forest Service's national research effort for forest tree improvement. By applying aseptic culture techniques to hybrid poplars it may be possible to identify and isolate variation for increased resistance to S. musiva in a much shorter period of time than would be possible in a conventional breeding program.

The specific objectives of this cooperative investigation between the NCFES, Forest Disease Project, and the Department of Horticultural Science and Landscape Architecture, University of Minnesota are: (1) the development of aseptic culture systems that allow plantlet regeneration at different levels of tissue organization and (2) the development of screening systems that can be used at either the cellular or plant level to identify and recover somatic variants that show increased resistance to S. musiva.

Plants regenerated from aseptic cultures of many agronomic species have expressed variation for a wide range of traits (Larkin and Scowcroft 1981). In the paper cited above any type of variation observed in plants obtained from aseptic culture systems was defined as "somaclonal variation".

The precise cause of observed variation has not been determined. In general, the variation can either be pre-existing in the explant material, as in sugarcane and potato (Brettell and Ingram 1979), or arise in vitro (Larkin and Scowcroft 1981). Factors that apparently contribute to somaclonal variation include the genetic background and source of the explant, growth medium, rate and method of transfer, time in culture, and characteristics of the aseptic plant culture such as growth rate (Orton 1983).

Possibly the most useful variant trait reported in regenerated plants is an increased resistance to various diseases (Heinz et al. 1977, Gengenbach et al. 1977, Matern et al. 1978, Sacristan 1982). The observed resistance in aseptic culture-derived plants was determined to be genetic in several instances (Gengenbach et al. 1977, Sacristan 1982) and maintained through several asexual generations in others (Heinz et al. 1977, Matern et al. 1978). Several sugarcane somaclones have been reported as being suitable for commercial plantings (Heinz et al. 1977).

Many reviews are now available which discuss the theory and techniques associated with obtaining disease resistant plants from aseptic culture systems (Chaleff 1983, Evans et al. 1984, Meredith 1983, Orton 1983). The ability to initiate and maintain cultures of the plant species under precisely defined conditions is essential, especially in the forms of suspension and plated cell cultures (Chaleff 1983, Meredith 1983). It is also important to know as much as possible about the pathogen, in particular, whether a culture filtrate that will induce symptoms of infection can be obtained (Brettell et al. 1979, Chaleff 1983, Meredith 1983). Finally, after resistant cells/cell colonies have been identified, the regeneration of whole plants must be possible.

There have been nearly 50 articles published on the aseptic culture of the genus Populus during the past 25 years. The first report of whole-tree regeneration from unorganized callus was by Winton (1970) and it is now possible for many poplar species and hybrids. The initiation and maintenance of suspension cultures (Winton 1968, Matsumoto et al. 1970) and subsequent plant regeneration (Riou et al. 1975, Douglas 1982) have also been reported. Variation in several morphological traits of putative P. x euramericana hybrid poplars regenerated from unorganized callus cultures was reported by Lester and Berbee (1977).

Screening for desirable traits in whole plants regenerated from aseptic culture systems can be accomplished by conventional methods. However, screening at the whole plant level is inefficient in terms of facilities and labor. Assuming that the trait for susceptibility or resistance to any particular pathogen is expressed at the cellular level, it would be much more efficient to screen callus (Helgeson and Haberlach 1980) or cell cultures (Chaleff 1983, Meredith 1983) prior to plant regeneration. It is important to realize that callus selection systems can be affected by many factors and may therefore be difficult to reproduce (Helgeson and Haberlach 1980). In addition, any type of callus selection system is likely to be inefficient when compared to suspension, and especially, plated cell selection systems (Meredith 1983).

Recent research has revealed that the potential productivity of many hybrid Populus clones is severely limited because of their extreme susceptibility to S. musiva (Ostry and McNabb 1983). Premature defoliation of trees predisposes them to other fungi and environmental stresses. Plantation failure has been caused by widespread tree mortality resulting from stem infections. The genus Populus is rich in genetic diversity, including resistance to S. musiva. However, most of the potentially highly productive clones tested thus far are too susceptible to this pathogen to be recommended for planting. Planting resistant clones is the most effective strategy for maximizing fiber yields.

SEPTORIA MUSIVA CULTURE AND FILTRATE ISOLATION

Isolates of S. musiva being used in this investigation have been recovered from infected hybrid poplars throughout the north central United States. Sporulating cultures are maintained on an agar-solidified medium at 20°C in a lighted incubator. Culture filtrates are being obtained from liquid shake cultures of the fungus grown in a defined medium. Filtrates are concentrated in a flash evaporator and partitioned against various organic solvents. Recovered fractions will be partially purified using adsorption chromatography.

POPULUS CULTURE SYSTEMS

In our work, callus has most often been obtained from the cut surfaces of stem internode, shoot tip, and leaf disc explants. The callus is initiated on agar-solidified Woody Plant Medium (WPM) (Lloyd and McCown 1980) in a dark incubator at a constant 25°C.

By manipulating the type, concentrations, and combinations of growth regulators in the nutrient medium a variety of callus types have been obtained. For example, callus initiated on a high 2,4-D: no cytokinin medium grows very rapidly, is difficult to maintain for extended periods of time, is very friable, and shows low organogenic potential. This type of callus is suitable for suspension culture inoculum. Meanwhile, callus grown on media containing approximately equal concentrations of NAA and BA also grows rapidly but is firm, easily subcultured at 4-6 week intervals, and maintains a high organogenic potential.

Callus initiated on a high 2,4-D medium must be transferred to a low 2,4-D medium for 30-60 days prior to transfer to a shoot proliferation medium. At present, WPM containing BA: low NAA is used to stimulate adventitious shoot formation and elongation from unorganized callus cultures of hybrid poplars in this study.

Adventitious shoots excised from callus cultures are rooted directly in a peat: perlite medium under high humidity conditions in a growth room. Rooting generally occurs in 14-21 days, at which time the plantlets are hardened off before being transferred to the greenhouse. Several hundred plantlets obtained from callus cultures of clones in this study have now been established in the greenhouse.

SCREENING METHODOLOGIES

At present, plants obtained from callus cultures, "somaclones", are being tested for variation in their susceptibility to S. musiva by means of a leaf disc bioassay (Spiers 1978) that has been modified for use under our conditions. Preliminary results of this initial screening process indicate that variation in resistance to the pathogen does exist. In addition, we are also using a leaf puncture assay to isolate those fractions of the culture filtrates that induce necrosis of leaf tissue. Identified toxic fractions may then be used to identify somaclones that express an increased resistance to S. musiva. It must be stressed that much work is still needed to eliminate all sources of extraneous variation from the assay procedures described above.

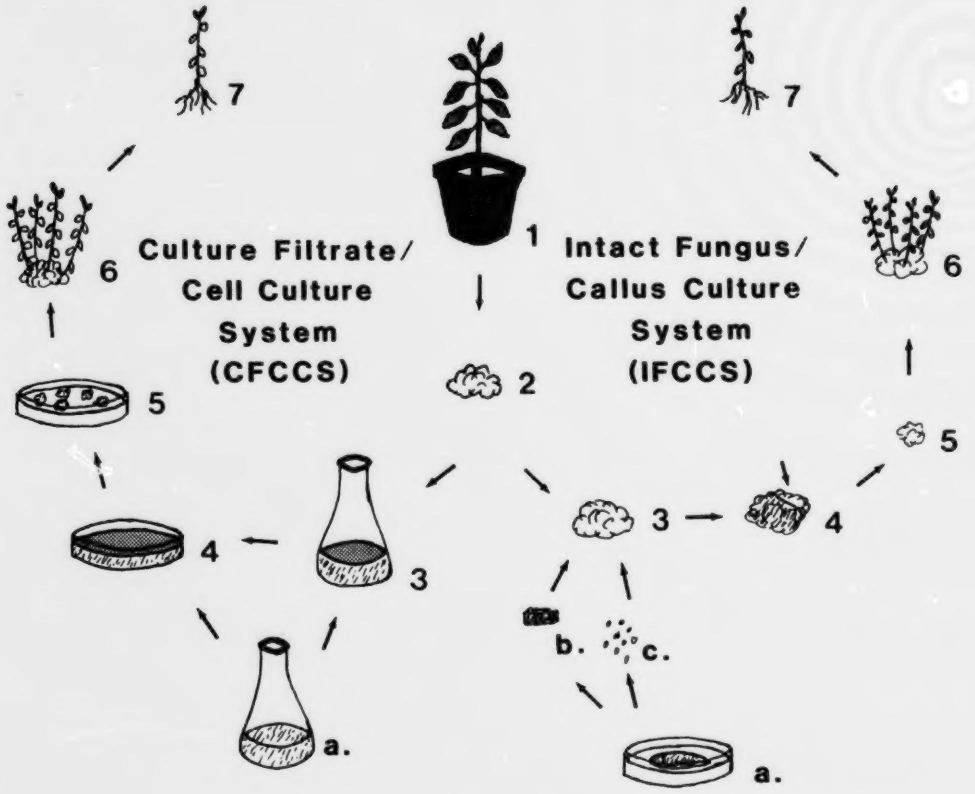
As previously mentioned, screening at the whole plant level is extremely inefficient. Therefore, we are developing in vitro screening systems as outlined in Figure 1. Plants regenerated from callus/cell colonies surviving exposure to various forms of S. musiva in vitro will be assumed to have an increased resistance to the pathogen. This assumption will be tested under greenhouse conditions, and ultimately, in the field.

SUMMARY

Methods for maintaining aseptic culture of S. musiva and several hybrid poplar clones have been refined. Further modifications may be necessary if indicated by continuing research findings.

Fungus and plant culture systems are to the stage of development at which screening of poplar regenerated from callus and cell cultures for variations in susceptibility to S. musiva can be initiated. In vitro screening methodologies are also being developed and should be available in the near future.

The benefit of this research will be to augment tree improvement programs so that high value planting material can be produced quickly. The ultimate benefit will be to remove the constraint of disease susceptibility to the production of maximum potential yields in intensively managed hybrid poplar plantations.


7
7
1
Culture Filtrate/
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b.
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a.

Figure 1. Diagrammatic representation of aseptic culture challenge systems for hybrid Populus. IFCCS: 1. Stock plant, 2. callus culture, 3. callus inoculated with mycelium (b.) or spores (c.) from S. musiva cultured on agar-solidified medium (a.), 4. callus resistant to pathogen (arrow), 5. isolated resistant callus, 6. shoot regeneration, 7. rooted plantlet to be tested for resistance.

CFCCS: 1. Stock plant, 2. callus culture, 3. Populus suspension culture and/or 4. plated cell culture with culture filtrate from shake culture of S. musiva (a.) added to the nutrient medium, 5. Populus cell colonies which survived exposure to filtrate, 6. shoot regeneration, 7. rooted plantlet to be tested for resistance.

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TESTING OF A METHOD FOR SCREENING ASPEN FOR HYPOXYLON CANKER RESISTANCE

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ABSTRACT

A random selection was made from 36 clones that had been tested *in vitro* for their resistance to *Hypoxylon* canker. The selected clones were vegetatively reproduced by bud culture. Three different growth media were used for comparative purposes. After transfer from stage II to stage III, each seedling from each clone was inoculated with a certain amount of an *Hyppoxylon mammatum*-specific toxin. Seedlings, reaction to toxin injection was quantified in order to establish a relation to their level of resistance in the field.

RÉSUMÉ

Une sélection aléatoire a été effectuée à partir de 36 clones qui avaient été testés *in vitro* pour leur résistance au chancre hypoxylonien. Les clones choisis ont été reproduits végétativement par la culture de bourgeons. Trois différents milieux de croissance ont été utilisés à des fins comparatives. Après le transfert du stage II au stage III, chaque semis de chaque clone a été inoculé avec une certaine quantité d'une toxine spécifique à *Hypoxylon mammatum*. La réaction des semis à l'injection d'une toxine a été quantifiée afin d'établir une relation avec leur niveau de résistance sur le terrain.

THE APPLICATION OF MOLECULAR BIOLOGY TO MASS CELL CLONING OF CONIFERS

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ABSTRACT

Very significant genetic improvement has been achieved by breeding of conifers. Additional restructuring of the genome will be possible in the future by genetic engineering as well as conventional breeding. Exploitation of these gains will require large-scale clonal propagation from single cells or protoplasts. Basic biological information is currently lacking on the molecular basis of gene activation during early embryogenesis. An understanding of the biochemistry of embryo formation from undifferentiated diploid cells following fertilization provides clues for induction of somatic embryogenesis. During a period of about 2 weeks after fertilization in Pinus strobus, the histogenesis of undifferentiated embryo initials is accompanied by transitions in RNA distribution in the ovule, and by the expression of genes for products of stage-specific messenger RNAs. These events precede differentiation in the zygotic embryo. They have important implications for the molecular manipulation of potentially active but strongly repressed genes affecting embryogenesis in somatic cells. A better understanding of repressor mechanisms and their manipulation may lead to the development of practical and economical technology for embryo induction in conifer cells and protoplasts.

Keywords: pine embryogenesis, somatic embryogenesis, messenger RNA, eastern white pine, Pinus strobus, cell culture, protoplast culture, cloning conifers.

RÉSUMÉ

Par la culture des conifères, on a pu obtenir une amélioration génétique très significative. À l'avenir, une restructuration additionnelle du génôme sera possible par génie génétique aussi bien que par croisement conventionnel. L'exploitation de ces gains va nécessiter une propagation clonale à grande échelle à partir de cellules isolées ou de protoplastes. Il y a présentement pénurie d'informations sur la base moléculaire de l'activation des gênes au début de l'embryogénèse. Pendant une période d'environ 2 semaines après la fertilisation chez Pinus strobus, l'histogénèse des éléments indifférenciés de l'embryon s'accompagne de transitions dans la distribution de l'ARN dans l'ovule, et par l'expression de gênes de produits d'ARNs messagers spécifiques à ce stade. Ces étapes précèdent la différentiation à

l'intérieur de l'embryon zygotique. Elles ont d'importantes implications pour la manipulation à l'échelle moléculaire de gènes potentiellement actifs mais fortement réprimés affectant l'embryogénèse dans les cellules somatiques. Une connaissance plus poussée des mécanismes de répression et de leur manipulation pourrait mener au développement d'une technologie pratique et économique d'induction de l'embryon dans les cellules et les protoplastes des conifères.

Mots-clés: embryogénèse du pin, embryogénèse somatique, ARN messager, pin blanc, Pinus strobus, culture cellulaire, culture du protoplaste, clonage des conifères.

DEVELOPMENT OF DOULGAS-FIR APICES UNDER NATURAL AND CONE-INDUCING CONDITIONS

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ABSTRACT

The anatomy, mitotic activity, size and total insoluble carbohydrate histochemistry were studied in axillary apices from 9- and 10-year-old Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) trees, after cone induction treatments of rootpruning (RP) and (or) stem injections of a gibberellin A_4 and A_7 ($GA_{4/7}$) mixture. Axillary buds were initiated at the time of RP, but RP had no effect on axillary bud initiation. Axillary apices from control and GA treated trees were similar and followed the normal sequence of bud-scale initiation, differentiation and leaf initiation described previously and no cone-buds differentiated. Early development of axillary apices from RP and RPGA treated trees was normal, but development became retarded near the time of vegetative bud flush. Retarded apices were small with low mitotic activity, and developed many features characteristic of latent apices. The ultrastructure of cells at the base of retarded apices showed dense cytoplasm and some unusual features. Apical retardation continued until mid-July when normal development resumed and apices differentiated into reproductive or vegetative buds, or became latent. The trees in which the greatest retardation of apical development occurred during lateral shoot elongation produced the most cone-buds. These results are discussed in relation to hypotheses proposed to explain how cultural and GA treatments affect cone induction in the Pinaceae.

Key words: conifer, apices, cone induction, bud differentiation.

RÉSUMÉ

Nous avons étudié l'anatomie, l'activité méiotique, la taille et l'histochimie des carbohydrates insolubles totaux dans les terminaisons apicales de douglas (*Pseudotsuga menziesii* (Mirb.) Franco) de 9 et 10 ans, après des traitements d'induction des cônes par la taille des racines et (ou) des injections d'un mélange de gibbérellines A_4 et A_7 ($GA_{4/7}$) dans la tige. Les bourgeons axillaires étaient déjà apparus au moment de la taille des racines mais celle-ci n'a pas eu d'effet sur l'apparition des bourgeons axillaires. Les terminaisons axillaires chez les témoins et les arbres traités au GA étaient semblables et suivaient la séquence normale précédemment décrite;

aucun bourgeon de reproduction ne s'est différencié. Le primo-développement des terminaisons axillaires des arbres traités par la taille seule et par la taille avec GA était normal mais leur développement subséquent fut retardé pratiquement jusqu'au moment du débourrement des bourgeons végétatifs. Les terminaisons retardées étaient petites, avec une faible activité méiotique, et développèrent de nombreux caractères propres aux terminaisons latentes. L'ultrastructure des cellules à la base des terminaisons retardées montra un cytoplasme dense et certains caractères inhabituels. Ce retard apical continua jusqu'à la mi-juillet: le développement normal repris alors et les terminaisons se différencièrent en bourgeons végétatifs ou reproductifs, ou devinrent latentes. Les arbres chez qui se produisit le plus long retard dans le développement des terminaisons pendant l'élongation des pousses latérales, produisèrent le plus grand nombre de bourgeons de de reproduction. Nous discutons de ces résultats par rapport aux diverses hypothèses proposées pour expliquer comment les traitements culturaux et les traitements aux GA affectent l'induction des cônes chez les Pinacées.

Mots-clés: conifère, terminaisons, induction des cônes, différentiation des bourgeons.

CONE INDUCTION IN WHITE AND NORWAY SPRUCE

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ABSTRACT

White spruce (Picea glauca (Moench) Voss) trees in a seed orchard were sprayed biweekly with 200 mg per liter of gibberellin $A_{4/7}$ ($GA_{4/7}$) under two different schedules. The optimal period for spraying was found to be from the time of bud break to that of bud differentiation for reproductive organs (May to July). Branches of some other trees were selected for spraying biweekly with 4 different concentrations of $GA_{4/7}$ from May to August. A significant increase in cone production was found on branches sprayed with 800 mg per litre of $GA_{4/7}$.

Girdling of Norway spruce (P. abies (L.) Karst.) trees resulted in a significant increase in cone production with an average of 72 cones per tree while cone production of the untreated trees averaged 34 per tree. However, yellowing of foliage was observed in the treated trees.

Keywords: Picea glauca, Picea abies, $GA_{4/7}$, Girdling, Seed Cone

RÉSUMÉ

Les épinettes blanches (Picea glauca (Moench) Voss) d'un verger à graines ont été arrosées toutes les deux semaines avec 200 mg par litre de gibbérelline $A_{4/7}$ ($GA_{4/7}$) selon deux calendriers différents. La période optimale pour l'arrosage semble aller du débourrement des bourgeons jusqu'à leur différentiation en organes reproducteurs (de mai à juillet). On a choisi les branches de quelques autres arbres pour les arroser toutes les deux semaines selon 4 concentrations différentes de $GA_{4/7}$ de mai à août. On a constaté une augmentation significative de la production de cônes sur les branches arrosées avec 800 mg par litre de $GA_{4/7}$.

Le cernage des épinettes de Norvège (P. abies (L.) Karst.) a donné une augmentation significative de la production de cônes avec une moyenne de 72 cônes par arbre alors que celle des arbres non traités était en moyenne de 34. Cependant, on a observé un jaunissement du feuillage des arbres traités.

Mots-clés: Picea glauca, Picea abies, $GA_{4/7}$, cernage, cône

INTRODUCTION

Application of gibberellin $A_{4/7}$ ($GA_{4/7}$) onto conifer trees can increase seed cone production if trees are treated at the appropriate time, proper duration, and suitable concentration (Tompsett et al. 1980, Chalupka 1981, Cecich 1983, Marquard and Hanover 1984). Harrison and Owens (1983) suggested that cone-inducing treatments be applied before differentiation. Marquard and Hanover (1984), and Cecich (1985) confirmed that $GA_{4/7}$ treatment initiated before meristematic differentiation enhanced seed-cone production in white spruce (Picea glauca (Moench) Voss). The same period of time was also reported to be best for Sitka spruce (P. sitchensis (Bong.) Carr.) grafts (Tompsett and Fletcher 1979). Chalupka (1981) observed significant promotion of pollen cones on Norway spruce (P. abies (L.) Karst.) grafts when they were sprayed with GA_3 in May and June and covered with polythene tents in July. However, there were great differences in clonal response to $GA_{4/7}$ treatment and between years in cone production (Dunberg 1980).

A close relationship exists between the end of shoot elongation and the time of cone-bud differentiation. The time of cone-bud differentiation can be quite accurately estimated by date of lateral shoot elongation (Owens and Molder 1977). White spruce trees begin to flush from early to mid-May and end shoot elongation by early July in the Lake States (Nienstaedt 1974). The present study was designed to 1) establish an optimal period of $GA_{4/7}$ spraying onto white spruce trees by using the calendar date, 2) define an optimal concentration of $GA_{4/7}$ solution for white spruce, and 3) determine the effect of girdling on Norway spruce.

MATERIALS AND METHODS

Experiment 1. Whole Tree Spraying. Forty white spruce trees representing 5 clones growing in a seed orchard in Midhurst, Ontario were selected for the study. The trees were 15-year-old grafts of the similar size within a clone. In May, June and July, 10 trees each were sprayed biweekly with either a solution containing 15% alcohol and 0.05% Tween 20, or a solution containing 200 mg/l of $GA_{4/7}$ in addition to alcohol and Tween 20. The other 20 trees had the same treatments in June, July and August. The foliage was thoroughly wetted with the solution until the solution was about to drop. The experiment was carried out for 3 years.

Experiment 2. Branch Spraying. Four branches of about the same size on the second whorl from the tree terminal were selected from each of 24 white spruce trees representing 6 clones. The grafts were 18 years old growing in the seed orchard. Foliage on the branches was thoroughly sprayed biweekly with either the control or the treatment solution from May to August for 3 years. The control solution contained 15% alcohol and 0.05% Tween 20, and the treatment solution either 200, 400, or 800 mg/l of $GA_{4/7}$ in addition to alcohol and Tween 20.

Experiment 3. Girdling. Two trees each from 11 Norway spruce clones were selected. They were about 15 years old growing in the Maple, Ontario spruce arboretum. Two half-moon overlapping girdles which were opposite each other and placed about 30 cm apart were carried out on each of the treated trees in early May, 1983. The width of the cut (2 mm) was about the thickness of the saw blade and the depth reached the inner bark to sever the phloem. The girdles overlapped about 3 cm on each cut end.

Data on seed cone production were collected in the year following the treatment. The number of whorls of branches bearing seed cones was counted from the terminal to the base of the trees. Analysis of variance and Duncan's multiple-range test were based on the outline by Steel and Torrie (1960).

RESULTS

Experiment 1. Whole tree spraying with $GA_{4/7}$ did not stimulate any cone crops in both 1983 and 1985. However, spraying in May, June and July in 1984 produced significantly more seed cones than the control while spraying in June, July and August was not effective (Table 1). Thirty-nine percent of the variation was attributed to treatments and 34% of the variation to clonal differences. Treatment and clonal interaction was not significant and constituted 17% of the variation.

There was a shift in the distribution of seed cones from the upper whorl to the lower whorl of branches. Trees sprayed in May, June and July in 1984 averaged 11.2 whorls bearing seed cones while the control had an average of 8.4 whorls producing seed cones (Table 1). No significant change was found on trees sprayed in June, July and August.

Experiment 2. Seed-cone production on all branches was poor in 1983 and 1985. In 1984, branches sprayed with 800 mg/l of $GA_{4/7}$ produced significantly more seed cones than those sprayed with either 0, 200 or 400 mg/l of $GA_{4/7}$ although the treated branches produced at least 20% more seed cones than the control. Clones and treatments accounted for 39% and 34% of the variation, respectively, while no interaction was found between clone and treatment (10% of the variation).

Experiment 3. Seed cone production was significantly increased by girdling. Girdled trees averaged 72 (± 36) seed cones per tree and non-girdled trees 34 (±44) seed cones per tree. The treatment accounted for 71% of the variation and clones for 26% of the variation. This treatment resulted in chlorosis in foliage but recovery occurred in the year following the cone production.

DISCUSSION

The timing of $GA_{4/7}$ application affected seed cone production, and the response occurred in a good, but not a poor crop year. In 1984, there was a good cone crop and the $GA_{4/7}$ spray significantly increased cone production (Table 1). Poor cone crops occurred in both 1983 and 1985, and $GA_{4/7}$ treatment did not stimulate cone production. Dunberg (1980) had a similar observation and found great differences between clones and between years in response to $GA_{4/7}$ treatment in Norway spruce. Puritch et al. (1979) concluded that $GA_{4/7}$ treatment could not guarantee good cone crops in Douglas-fir (Pseudotsuga menziesii (Mirb.) Franco).

Spraying white spruce trees with $GA_{4/7}$ from May to July significantly increased seed-cone production while spraying from June to August was ineffective (Table 1). Cecich (1985) reported that elongating shoots sprayed from May 13 to June 3 produced significantly more seed and pollen cones than did elongating shoots sprayed from June 10 to July 1. Marquard and Hanover (1984) emphasized that $GA_{4/7}$ treatment should be initiated before meristematic differentiation began (late June). Tompsett and Fletcher (1979) applied $GA_{4/7}$ underneath the bark flaps to Sitka spruce and found that application in May and June was more effective than in July and August. The present study suggested that $GA_{4/7}$ treatment should begin during bud scale initiation and before meristematic differentiation in the buds. The bud types may be determined biochemically during the process of bud scale initiation. According to Owens and Molder (1977), pollen- and seed-cone apices produced much less bud scales than vegetative apices in white spruce.

Seed-cone production varied with concentration of $GA_{4/7}$ treatment (Tompsett *et al.* 1980). Application of 200 mg/l of $GA_{4/7}$ to white spruce was effective in whole tree spraying from May to July while in branch spraying, the concentration was only 20% better in seed-cone production than the control (Table 2). Spraying with 800 mg/l of $GA_{4/7}$ was significantly better than the control. Marquard and Hanover (1984) found that shoots sprayed with 250 mg/l of $GA_{4/7}$ bore significantly more seed cones than those sprayed with 500 mg/l. Cecich (1985) used 600 mg/l of $GA_{4/7}$ spray to effectively stimulate seed-cone production. Chalupka (1979, 1981) reported that application of 100 mg/l of GA_3 to Norway spruce in May and June significantly increased the pollen-cone production and a synergistic effect was observed when GA_3 was applied with either chlorcholinchloride in mid-June, or polythene covers in July. Cecich (1983) applied 3 concentrations of $GA_{4/7}$ (200, 400, and 600 mg/l) to jack pine (*Pinus banksiana* Lamb.) and found the best spray was 600 mg/l of $GA_{4/7}$.

Table 1. Average number of seed cones per tree and number of whorls bearing seed cones on white spruce trees sprayed with $GA_{4/7}$ solution in two periods

	Average number of seed cones per tree			Average number of whorls having seed cones
	1983	1984	1985	1984
Control	0	1647±799	0.7±2.2	8.4±1.6
May-June-July	10.6±16.0	3051±1394*	13.0±39.7	11.2±1.3**
Control	0.3±0.5	2947±1641	0.4±1.3	9.7±2.5
June-July-August	13.5±34.7	2749±1054	4.0±12.6	10.6±1.6

* differ significantly at the 5% level.
** differ significantly at the 1% level.

Table 2. Average number of seed cones per branch on branches sprayed with different concentrations of $GA_{4/7}$ from May to August in white spruce. The data in brackets indicate the total number of cones

	Average number of seed cones per branch		
	1983	1984	1985
Control	(2)	149.0±94.0 a*	(0)
$GA_{4/7}$ 200 mg/l	(2)	179.7±71.7 a	(4)
400 mg/l	(7)	187.2±74.0 a	(0)
800 mg/l	(5)	204.3±84.5 b	(0)

* means followed by the same letter are not significantly different.

Gradients of plant growth regulators have been proposed as a mechanism in controlling the zonation of seed- and pollen-cone production within the crowns of many conifer species (Ross 1976, Pharis _et al._ 1980). Marquard and Hanover (1984) observed a shift of seed-cone distribution to more distal shoot positions in $GA_{4/7}$ sprayed branches in white spruce. This was attributed to the chemical gradients within individual shoots. In the present study, the sprayed trees shifted their seed-cone production to the lower crowns (Table 1). The spray of $GA_{4/7}$ may have resulted in altering the chemical level in the lower crown.

Wounding treatments have been successfully used to induce cone production. Melchior (1960) found that girdling enhanced cone production in the following year in European larch (_Larix decidua_ Mill.). Ebell (1971) established the optimal time to be about one month before vegetative bud break for girdling in Douglas-fir. Both annual and biennial girdle regimes resulted in improved cone yields in four successive years (Wheeler _et al._ 1985). Girdling in early May in Norway spruce significantly increased seed-cone production but chlorotic foliage resulted from girdling. The chlorosis may be corrected by the application of nitrogen fertilizer.

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EVALUATION OF ULV SPRAYS FOR SEED ORCHARD APPLICATION OF $GA_{4/7}$

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ABSTRACT

Gibberellin A4/7 mixture (GA4/7)[1] has been shown to be an effective treatment for enhancing cone production in Douglas-fir and western hemlock. Foliar spray by high volume (HV) sprayer has been shown to be less effective in Douglas-fir than stem injection, and is also quite wasteful of this relatively expensive chemical.

This paper reports on the use of ultra low volume (ULV) spray equipment for the application of GA4/7 as a foliar spray to seed orchard trees of Douglas-fir and western hemlock. At the rates applied in these studies, the ULV equipment was more successful than the HV on Douglas-fir, but the reverse was true for western hemlock. The GA dosage applied by ULV appeared to be sub-optimal in the latter species.

Keywords: cone induction, gibberellins, seed orchard management

RÉSUMÉ

Le mélange de gibbérellines A4/7 (GA4/7) est apparu comme le traitement le plus efficace pour augmenter la production de cônes chez le douglas et la pruche de l'Ouest. La pulvérisation sur les feuilles au moyen d'un gicleur à grand volume est apparue moins efficace sur le douglas que l'injection dans la tige et elle est très peu économe de ce produit chimique relativement coûteux.

Ce texte décrit l'emploi d'un équipement de pulvérisation à très petit volume pour appliquer le GA4/7 sur le feuillage des douglas et des pruches de l'Ouest dans un verger à graines. Aux taux appliqués dans ces études, l'équipement à très petit volume a eu plus de succès que l'équipement à grand volume sur le douglas mais c'est l'inverse pour la pruche, le dosage appliqué par petit volume semblant être sous-optimal pour cette dernière espèce.

Mots-clés: induction de cônes, gibbérellines, gestion des vergers à graines.

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[1] Correct usage: GA4/7.

INTRODUCTION

Gibberellin A4/7 mixture (GA4/7) has been shown to be an effective treatment for enhancing cone production in Douglas-fir (Pseudotsuga menziesii (Mirb.) Franco) and western hemlock (Tsuga heterophylla (Raf.) Sarg.). Foliar application by high volume (HV) mist sprayer has been shown to be less effective in Douglas-fir than stem injection (Ross, unpublished data). High volume mist sprayer is the normal mode of application for GA4/7 to western hemlock. This mode of application is quite wasteful of this relatively expensive chemical.

In pesticide applications, ultra low volume (ULV) spray equipment has been found to give the same or better biological control with less chemical than required with HV sprays (Baldwin and Stanley 1981). This greater efficiency of ULV sprayers is due to several factors. HV mist sprayers produce a wide range of droplet sizes (6 to 700 μm), the smaller of which (under ca. 30 μm) drift and rapidly evaporate, while the larger (above ca. 200 μm) are lost to gravity or result in overkill. With ULV spray equipement the droplet size is controlled within a narrow range (ca. 60 to 110 μm) to minimize these losses. The smaller-sized droplets remain suspended in the air stream for some time where they are carried in eddy currents created within the crown, thus giving better target coverage, especially on the underside of leaves. And, the smaller droplets are also more efficiently absorbed as a result of their higher surface area to volume ratio.

Foliar absorption can be further improved by the use of horticultural spray oils which facilitate the penetration of chemicals through stomata and the lipophyllic cuticle of leaves and new shoots (Baldwin and Stanley 1981, Johnson 1982). Spray oils, in addition, have anti-evaporant and pesticidal (on arthropods) properties, and their use also improves spray dispersal. The superior horticultural spray oil is a highly refined, light weight (60 to 70 second viscosity) paraffinic oil, with an unsulfonated residue of 92% or greater, and a 50% distillation range at 10 mm Hg of 420 ± 8°F (Johnson 1982).

Although horticultural spray oils have been recommended for use undiluted on some crop and horticultural species (Baldwin and Stanley 1981), Ross (1983) observed that Douglas-fir, western hemlock and white spruce (Picea glauca (Moench) Voss) trees may be injured at oil concentrations as low as 5% in water. According to Johnson (1982), most woody plants can tolerate 2% of oil if healthy and not subjected to severe water stress. Sunspray 6E manufactured by Sunoco, was selected for testing because of the manufacturer's assurance of low potential phytotoxicity.

The two studies described in this paper were designed to test the effectiveness of ULV spray equipment for the application of GA4/7 as a foliar spray to seed orchard trees of Douglas-fir and western hemlock. Both studies were conducted at MacMillan Bloedel Limited's Harmac Tree Improvement Centre near Nanaimo, British Columbia.

MATERIALS AND METHODS

Douglas fir

This study involved 8-year-old rooted cuttings and was initiated in the spring of 1983. The design was a randomized complete block with six replications of four trees each assigned to nine treatments:

1: Ungirdled Control
2: Girdled Control
3: HV, Girdled + 400 mg/l GA4/7[2] + 0.05% (W/V) Aromox C12W surfactant[3]
4: ULV, Girdled + 800 mg/l GA4/7 + 0.05% (W/V) Aromox C12W surfactant
5: ULV, Girdled + 1600 mg/l GA4/7 + 0.05% (W/V) Aromox C12W surfactant
6: ULV, Girdled + 3200 mg/l GA4/7 + 0.05% (W/V) Aromox C12W surfactant
7: ULV, Girdled + 800 mg/l GA4/7 + 0.05% (W/V) Aromox + 2% Sunspray 6E Oil[4]
8: ULV, Girdled + 1600 mg/l GA4/7 + 0.05% (W/V) Aromox + 2% Sunspary 6E Oil
9: ULV, Girdled + 3200 mg/l GA4/7 + 0.05% (W/V) Aromox + 2% Sunspray 6E Oil

All girdled trees received overlapping, half-circumferential girdles using a sharp pruning saw (Wheeler *et al.* 1985). Trees were girdled in the spring once the vegetative buds had visibly swollen but before flushing had occurred.

Gibberellin foliar sprays were initiated when approximately 50% of the trees had flushed, and were repeated once weekly for six weeks. A Turbair Sprite, fan-assisted sprayer was used for the ULV applications, and a standard hand-pump, backpack, mist sprayer was used for the HV applications.

The volume of solution applied per tree by ULV sprayer (15 ml) was found to be approximately 1/20 of that applied by the HV sprayer (240 ml). At the selected ULV spray concentrations of 800, 1600, and 3200 mg/l, the amount of GA4/7 received per tree was equal to about .1x, .2x, and .4x, respectively, that received by the HV sprayer with GA4/7 at 400 mg/l.

Western Hemlock

This study, which was initiated in the spring of 1984, involved 12-year-old rooted and grafted ramets of western hemlock. One ramet from each of 24 clones was randomly assigned to receive one of four treatments:

1: Untreated Control
2: HV, 200 mg/l GA4/7 + 0.5% (W/V) Aromax C12W surfactant
3: ULV, 175 mg/l GA4/7 + 0.5% (W/V) Aromax C12W + 2% Sunspray 6E oil
4: ULV, 875 mg/l GA4/7 + 0.5% (W/V) Aromax C12W + 2% Sunspray 6E oil

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[2] ICI Plant Protection Division, Fernhurst, Haslemere Surrey, England (Lot #4068/116/2, 35.6% A_4: 60.4% A_7).

[3] Armak Chemical Limited, 100 University Avenue, Toronto, Ontario, M5J 1V6.

[4] Sun Oil Company.

Gibberellin A4/7 foliar sprays were initiated at vegetative bud burst, and were repeated once weekly for six weeks. A Turbair Fox[5], fan assisted sprayer was used for the ULV applications, and a standard handpump, backpack, mist sprayer for the HV applications. At the selected ULV spray concentrations of 175 and 875 mg/l, the amount of GA4/7 received per tree was equal to about 0.1 times and 0.5 times, respectively, that received by the HV sprayer at 200 mg/l.

RESULTS

Douglas-fir

Flowering responses were assessed in the spring of 1984. As the trees used varied greatly in size and crown form, counts of seed-cone buds were expressed on a per-branch basis: one branch per whorl in the uppermost five whorls was sampled, this being the maximum number of whorls possessed by all trees. Pollen cones were scored for each branch as:

0 = nil, 1 = light, 2 = moderate, and 3 = heavy production

Results are presented in Figure 1.

Trees in all treatments produced abundant pollen-cone buds as a result of the adjunct stem-girdling treatments. The pollen response was independent of GA treatment.

In terms of female flowering, the trees also responded well to the adjunct stem-girdling treatment which tended to mask the effects of applied GA4/7. Girdled controls averaged 2.3 seed-cone buds per branch, versus 0 for the 14 totally untreated trees. This production exceeded that (1.3 cones/branch) in response to the mist-spray treatment with GA4/7 at 400 mg/l^{-1} in water containing Aromax C12W surfactant (water-surfactant formulation). This particular treatment was chosen on the basis of being at least marginally effective in several previous trials on Douglas-fir, and what appears to be an inhibitory effect in the present study may in fact be a sampling problem.

The same water-surfactant formulation was more effective at 0.2 the dosage of GA4/7 when applied with ULV than mist sprayer (3.7 vs 1.3 cone-buds/branch). At 0.1 the dosage, this treatment was no better than girdling alone. Addition of 2% (v/v) Sunspray 6E horticultural spray oil significantly enhanced the efficacy of GA4/7 applied by ULV spray. Trees in these treatments produced an average of 168% more seed-cone buds each than those which received the same concentrations of GA4/7 as a water-surfactant ULV spray without oil. Compared to the mist-spray treatment, the water-surfactant-oil sprays resulted in 4.2 fold more seed-cone buds at only 0.1 the dosage of GA4/7. GA4/7 concentration did not increase the female response. In fact, the highest concentration (3200 mg/l) appeared to be supraoptimal. Furthermore, none of the phytotoxic effects observed in previous trials (see Introduction) were evident where spray oils were used at much higher concentrations.

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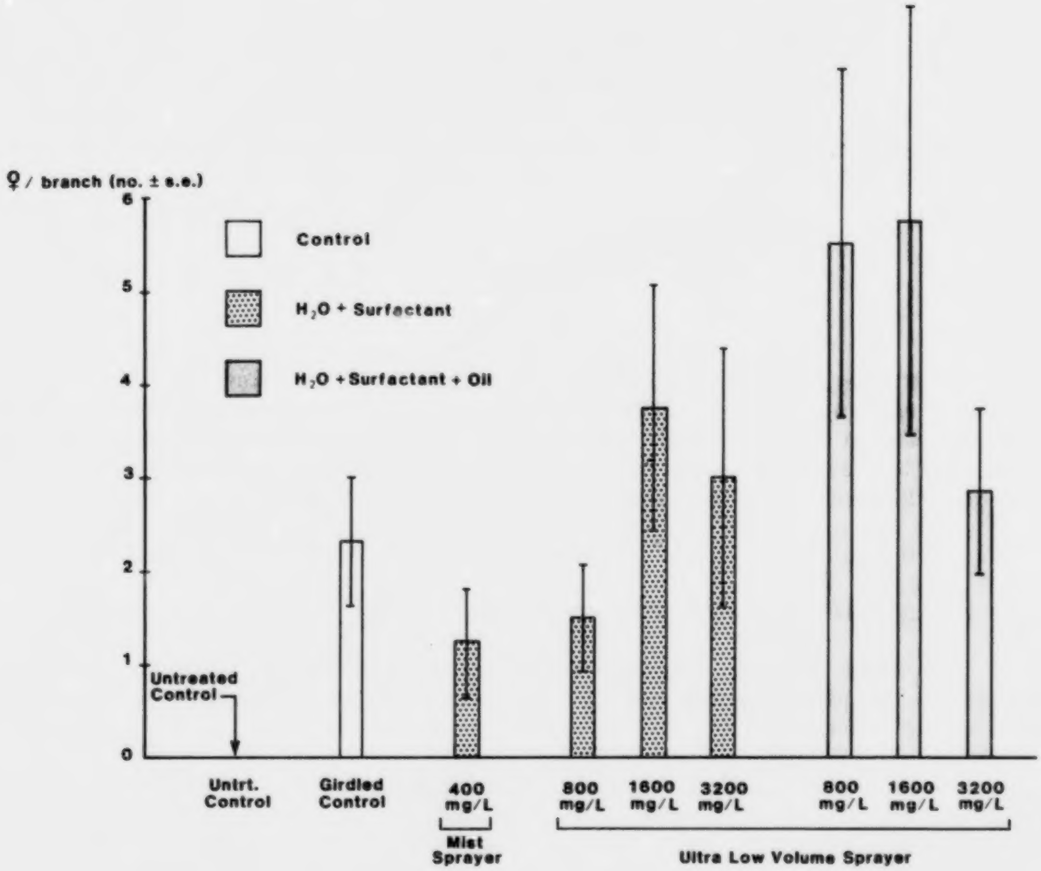
[5] London Fog Company, Long Lake, Minn., U.S.A.



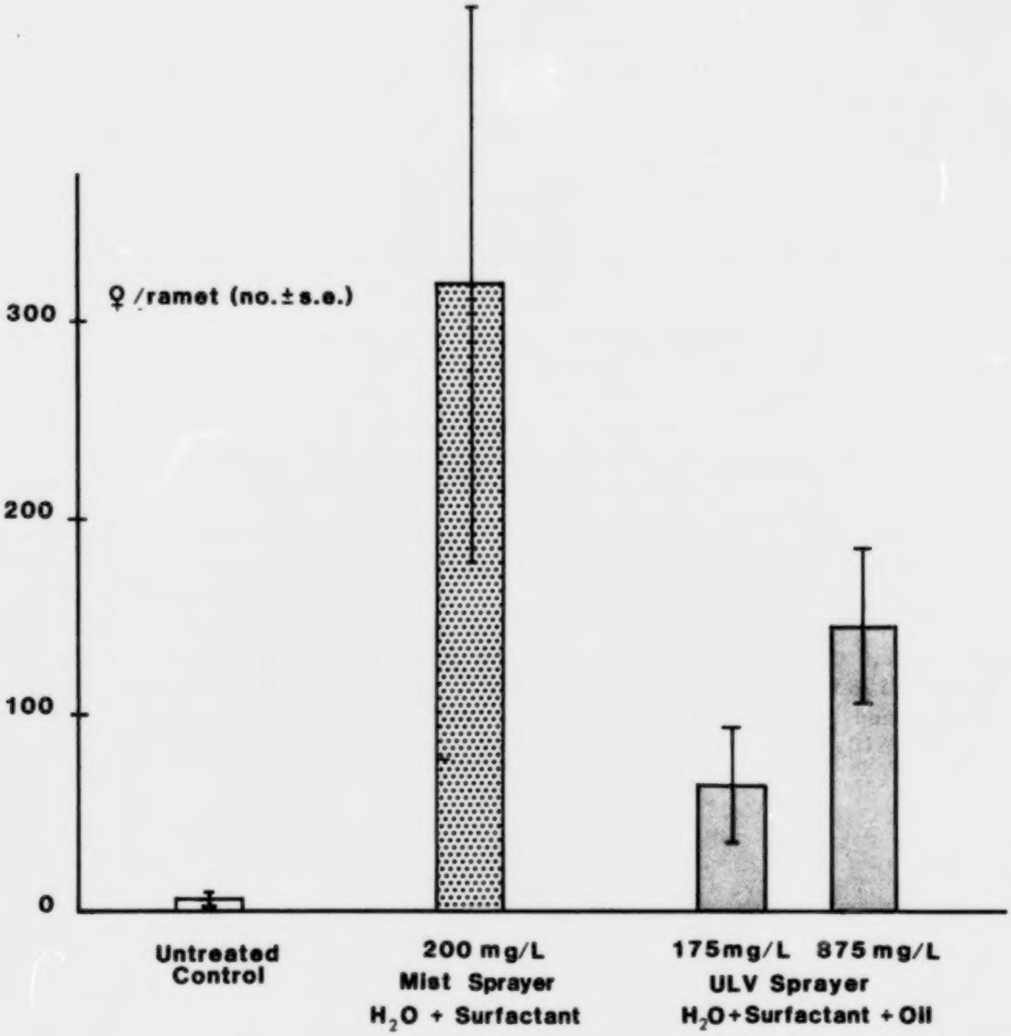
Western Hemlock

Flowering responses were assessed in the spring of 1985. The response to treatment was not as dramatic as for Douglas-fir. Therefore, whole-tree counts of seed-cone buds were made, and pollen-cone buds were scored on their presence or absence (0 or 1).

At the rates applied in this study, the HV spray treatment was more effective than either of the ULV treatments (Figure 2). For the HV treatment, 100% of the trees produced at least some seed cone buds, as compared to 88% for the 875 mg/l ULV, 35% for the control and 33% for the 175 mg/l ULV. The high concentration ULV (875 mg/l) appears to still be suboptimal in concentration. The large amount of variation seen in the size of the standard errors is the result of both site and genetic differences.

The HV and 875 mg/l ULV treatments produced slightly more pollen cone-buds than the control and 175 mg/l ULV (0.6 and 0.7 vs. 0.4 and 0.3). As well, a higher percentage of the trees produced pollen-cone buds with these treatments (61% and 71% vs. 35% and 33%).

Both seed-cone bud and pollen production in this study were much lower than in smaller potted material that received drought stress (unpublished data). It is felt that the response in this study could have been improved if an adjunct stress-inducing treatment had been applied.



CONCLUSIONS

It appears that ULV spray equipment is an effective method of applying GA4/7 as a foliar spray to Douglas-fir. A surfactant and a horticultural oil are necessary additions.

High volume spray equipment was more effective for use with western hemlock. The highest ULV concentration appeared to be suboptimal. There is a need for further work with western hemlock to determine the effectiveness of higher concentrations of GA4/7, as well as the effectiveness of adjunct stress-inducing treatments.

The use of ULV spray equipment does, however, have practical constraints. Due to the very small droplet size, even a slight wind causes drift. For maximum effectiveness of the chemical, it is desirable to have a minimum of eight hours without rain after spraying. No wind and no rain are a combination of conditions that do not frequently occur on the coast of British Columbia.

ULV sprays could have practical application in an indoor potted orchard (Ross *et al.* 1985).

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Fig. 1. Female flowering response by girdled 8-year-old Douglas-fir rooted cuttings to different spray treatments of gibberellin $A_{4/7}$ (24 trees per treatment).

Fig. 2. Female flowering response by 12-year-old western hemlock trees to different spray treatments of gibberellin $A_{4/7}$ (1 rooted or grafted ramet from each of 24 mature clones per treatment).

ESTIMATING SUPPLEMENTAL MASS POLLINATION (SMP) SUCCESS ELECTROPHORETICALLY

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ABSTRACT

Electrophoretic analyses demonstrated that supplementally applied pollen can be easily and accurately detected, even when competing with pollen from over 200 orchard clones, when pollen parents with appropriate multi-locus genotypes are selected. SMP efficacy, averaging 45%, was influenced significantly by cone-bud phenology at time of treatment and the number of treatments a tree received. Pollen dilution did not significantly reduce SMP success. Supplemental pollination appears promising as an operational tool in Douglas-fir seed orchards.

Keywords: Supplemental mass pollination, seed orchard, allozymes

RÉSUMÉ

Des analyses électrophorétiques ont démontré que le pollen appliqué en supplément peut être aisément et exactement détecté, même en présence de pollen de plus de 200 clones de verger, quand on sélectionne des parents polliniques avec des génotypes à loci multiples appropriés. L'efficacité du pollen appliqué en supplément, de 45% en moyenne, est influencée directement par la phénologie du bourgeon de cône au moment du traitement et le nombre de traitements qu'un arbre a reçu. La dilution du pollen n'a pas significativement réduit le succès du pollen appliqué en supplément. Cette pollinisation semble prometteuse comme outil d'aménagement dans les vergers à graines de douglas.

Mots-clés: pollinisation supplémentaire en masse, verger à graines, allozymes

INTRODUCTION

The function of a seed orchard is to provide a sustained and adequate yield of genetically improved seed. This paper is primarily concerned with the genetic quality aspects of the seed orchard function. Specifically, the paper addresses the use and evaluation of supplemental mass pollination (SMP).

The extent to which the genetic potential of a wind-pollinated seed orchard is realized has been referred to as the orchard's "efficiency" (Woessner and Franklin, 1973) or more recently, "genetic efficiency" (Adams and Joly, 1980; El-Kassaby et al., 1984). To obtain full or maximum genetic efficiency in an orchard, several conditions must be met: (1) all clones (or families) contribute equally to the gene pool, (2) clonal pollen-shed and seed-cone receptivity are essentially synchronized throughout the orchard, (c) pollen contamination from outside sources is negligible, (d) all possible crosses are equally compatible and (e) self-fertilization in orchard trees is negligible (Eriksson et al., 1973; Woessner and Franklin, 1973). In short, if orchard seed is to reflect its theoretical expectation of genetic gain the orchard itself must represent a nearly perfect, closed, panmictic population. Most orchard managers are aware that these conditions are seldom, if ever, met; condition violations are well documented (Eriksson et al., 1973; Jonsson et al., 1976; Shaw and Allard, 1982; Griffin, 1980; O'Reilly et al., 1982; Smith and Adams, 1983; Omi, 1983).

In 1973, Woessner and Franklin proposed using supplemental mass pollination in operational seed orchards to help reduce genetic gain losses due to the above-mentioned condition violations and to increase yields of filled seed. Subsequently, Denison and Franklin (1975) suggested that supplemental mass pollination would ultimately become an integral part of the seed orchard management system for most of those species that produce copious quantities of pollen. Indeed, a number of attempts to use SMP on orchard-size trees have been made (van der Sijde, 1971; Daniels, 1978; Webber, 1981; Bridgwater and Bramlett, 1982; Bridgwater and Williams, 1983; Hadders, 1984). These and other studies detailed in a review by Bridgwater and Trew (1981) have, by and large, relied on filled seed count and cone survival as indicators of SMP success. Such measures are not directly equatable with fertilization success of supplementally applied pollen and, in fact, may be quite misleading. Studies with dyed pollen (Bridgwater and Williams, 1983) are also informative but still ambiguous. Only by directly determining the paternal contribution of fertilized seed can one estimate the success of a supplemental pollination with certainty. The best technique currently available for doing this is starch-gel electrophoresis (Joly and Adams, 1983).

The purpose of this paper is (1) to demonstrate the use of allozyme markers (electrophoresis) in estimating SMP efficacy and (2) to review the results of our early attempts at operational SMP in a mature, Douglas-fir seed orchard.

MATERIALS AND METHODS

Study Trees

Study trees were located in the Coos Bay and Springfield orchard blocks at the Weyerhaeuser Seed Orchard near Turner, Oregon. Trees in these orchards averaged 15 years of age and 12-14 meters in height at the time of treatment. Each orchard block was roughly five hectares in size and contained some 900 trees representing 100 clones.

One ramet was selected from each of three clones to receive SMP treatment using fresh pollen from a single male parent. All four clones were chosen on the basis of their allozyme genotypes (Table 1), with female clones being primarily homozygous for common alleles at an array of loci and the male clone either homozygous or heterozygous for a number of low frequency alleles at these same loci. Clone 76, chosen as the pollen parent, had three pollen-producing ramets in the test orchard. Of the three clones chosen as females, two incurred considerable mortality and were discarded from this study. The remaining female, clone 53, resides in the same block as the pollen parent. However, since the Coos Bay and Springfield blocks are in close proximity (1/3 km) and could potentially share pollen pools, allele and genotype frequencies for the two blocks have been combined (Table 1).

Table 1: Allozyme genotypes of SMP test clones for the five most diagnostic loci studies. Genotype frequencies for the combined Coos Bay and Springfield orchards are shown in parentheses; allele frequencies are noted at the bottom of the table

	Genotype at Locus[1]				
Clone	PGM	CAT	IDH	6PGD	UGPP2
♀ 53	1/1 (.692)[2]	1/2 (.467)	1/1 (.574)	1/1 (.846)	2/5 (.067)
♂ 76	1/2 (.195)	2/2 (.077)	2/2 (.067)	1/5 (.092)	5/5 (.015)
Allele					
1	.828	.667	.740	.921	---
2	.120	.295	.224	---	.330
5	---	---	---	.051	.087

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[1] Genotype designations refer to the allelic constitution at that locus as arbitrarily assigned according to allozyme mobility.

[2] Genotype frequencies noted are for the observed genotypes of the study clones only. Allele frequencies are given only for the alleles possessed by study clones. They need not sum to 1.0.

Pollen Collection and Processing

Pollen cones were stripped by hand into large kraft bags soon after natural pollen shed had commenced. When 20 to 30 liters of buds had been collected they were transferred to a large-lot pollen processor. Pollen was extracted at approximately 10% moisture content after 36-48 hours. About 1.5 liters of clone 76 pollen was processed over a period of seven days. Germination counts on agar indicated pollen was alive but pollen vigor was not determinable.

Study Approach

Four branches, each with at least 25 seed-cone buds, were chosen in the mid- to upper-crown of each female clone. Buds were individually tagged and phenologically assessed on a daily basis throughout the period of receptivity. Each of the four branches on a tree received a different SMP treatment:

Branch 1: Pure pollen (76), single SMP event
Branch 2: Pure pollen (76), two SMP events, 2-3 days apart
Branch 3: Pollen mixed 1:1 with dead pollen, single SMP event
Branch 4: Pollen mixed 1:1 with dead pollen, two SMP events

Branches were supplementally pollinated when approximately 50% of the seed-cone buds in the mid- and upper-crown were receptive. Individual buds were phenologically assessed as being early (essentially non-receptive at time of SMP -- occasionally cone bracts broke through bud scales), prime (1/4 to 3/4 exposed, for less than three days) and late (receptive three or more days when pollinated). Late flowers are still within the optimal time of pollination for Douglas-fir. Branches treated twice received their second SMP two to three days after the first. Frequently, cones labeled early at the time of the first pollination were considered prime at the time of the second pollination.

Pollen Delivery

Pollen was delivered to receptive flowers using a Weyerhaeuser-designed pollinator consisting of a blower, a pollen hopper and an auger system which metered the pollen into the air stream at desired rates. The pollen-stream was directed at individual cone buds or bud clusters. For this experiment, pollen was delivered at the same rate to all branches. Individual branches were protected from the other treatments only by physical separation (3 to 5 meters).

Electrophoresis

Enzyme designations, buffer systems, stain recipes and starch-gel electrophoretic techniques used in this study have been described previously (Harris and Hopkinson, 1976; O'Malley et al., 1980; Conkle et al., 1983; Jech and Wheeler, 1984).

Clone genotypes were inferred using 7 to 10 megagametophytes per clone. All clones in the two orchard blocks (over 200) were genotyped. Of the 31 loci assayed for in megagametophytes, 15 were reliably detectable in embryos and five of these were particularly useful for designating paternity (Table 1) due to the rare combination of alleles possessed by the pollen parent.

In the fall of 1984, SMP cones were individually collected and processed. Filled seed counts were determined by x-ray analysis. Germinated seeds were dissected when radicles were 3-5 mm long, and megagametophytes and embryos were analysed side by side on starch gels. Paternal contribution to the embryo was inferred by subtracting the female contribution detected in the megagametophyte from the diploid embryo genotype. The total number of seed analysed were distributed among phenological classifications as follows: early 124; prime 383; late 529 and control 60 (from non-SMP cones located on same ramets).

Interpretation

Individual seed were identified as having been fertilized by supplementally applied pollen or by windborne pollen based on observed multilocus paternal genotypes. A seed was considered to be in one of three categories: (1) fertilized by windborne pollen if it possessed at least one paternal allele not found in clone 76 (e.g., allele 1 for IDH), (2) "probable success" (fertilized by clone 76) if it possessed the three locus paternal genotype 2-2-5 (allelic designations) for the CAT, IDH and UGP2 loci, or (3) "unambiguous success" if, in addition to the above, the seed possessed an allele 2 for PGM and/or allele 5 for 6PGD since only clone 76 possessed such a potential gamete producing genotype. The three-locus paternal genotype was considered "probable" because three other clones could have produced it also (one in Coos Bay and two in Springfield blocks), although none of these were homozygous for the low-frequency allele at all three loci such as clone 76. Considering the number of flowering ramets from these three clones and their proximity to female test clones, it is highly improbable (<1%) that they would be contributing significantly to the windborne pollen pool.

Analyses

Three variables were tested in a complete factorial design: pollen purity (dilute or pure), number of applications (one or two) and phenology of cone at time of treatment (early, prime or late). The experimental unit was considered the proportion of SMP fertilized seed as determined on a per cone basis. Experimental unit values were determined by analysing between 5 and 30 seed per cone ($\bar{x}$ = 15). Between 2 and 14 cones were analysed per factorial treatment combination ($\bar{x}$ = 8). The unbalanced data set was analysed using the general linear models (GLM) package of SAS (1982). This provided for estimates of the three main effects, three two-way interactions and one three-way interaction. Filled seed, total seed (empty + filled) and percent filled seed were calculated on a per cone basis for the same set of cones. These values, based on hand-extraction of all seed, were analysed according to the procedure just described.

RESULTS AND DISCUSSION

Two of the three ramets receiving SMP suffered significant cone-bud mortality soon after treatment. While some of the abortion was clearly due to an untimely frost, most appeared to be in response to the treatment itself. Two factors seemed to be responsible. One clone was pollinated soon after a rain when cone buds were still moist; studies in British Columbia suggest this can lead to increased abortion (Karlsson, 1981). Secondly, the force of the air-blast delivering the pollen was greater than necessary. We believe the buds may have actually suffered mechanical damage by the force of the SMP blast. The remaining ramet, of clone 53, suffered less than 25% abortion and it is from this clone alone that all data were derived for this paper.

Overall, 45% of the seed from cones that were receptive at the time of SMP were fertilized by delivered pollen. Success of SMP (reported as combined "probable" and "unambiguous" seed) varied significantly by number of applications ($p = .027$), and phenology condition of cone ($p = .001$; Tables 2 and 3).

Phenology was the more important factor affecting SMP success in this study. Average success rate with prime flowers was 54%, compared to late flowers with 38%. While this latter value is considerably lower, it is still encouragingly high. It demonstrates that cone buds need not be pollinated immediately upon becoming receptive in order to get positive results. This is important since phenological variation within a tree can be appreciable (Wheeler, unpublished data), preventing simultaneous treatment of all cone-buds at the same stage of receptibility. The 29% success rate for early cones is deceivingly high; it is inflated by the double treatment values (Table 2). For double treatments, these cones were rated as early the first treatment but most were prime by the second treatment. Thus, there is a logical confounding of main effect. This was further demonstrated by the statistically significant interaction between number of applications and phenology ($p = .05$). Values for early clones can only be interpreted directly from single application treatments.

Two applications were more effective than one (46% versus 39%) but the advantage was in hitting more cone buds at full receptivity, not in hitting the same bud twice (Table 2). Pure pollen was more effective (46% versus 41%) than diluted pollen but the difference was not large or statistically significant. Consequently, the use of expensive pure pollen for large scale pollinations is probably not warranted. This supports earlier work suggesting pollen dilution does not hinder seed set (Owens _et_ _al._, 1981; Daniels, unpublished data).

Filled seed per cone roughly paralleled the results observed electrophoretically. Phenology was the only factor significantly influencing filled seed; early cones having the fewest, late cones the most (Table 3). The interesting comparison is with control cones collected elsewhere in the crown, away from direct SMP areas. Filled seed in these cones was approximately half that of SMP cones (Table 3).

The success of this semi-operational SMP event can be further judged in light of the production orchard conditions existing at the time of treatment. Over 40% of orchard ramets flowered in 1984, with an average of nearly 2000 seed-cones per bearing tree. The pollen crop was similarly large, presumably resulting in considerable competition for fertilization rights. The female clone in this study was phenologically intermediate relative to all other orchard clones so a significant pollen source was available.

Table 2: Mean number of filled seed per cone ($\bar{x}$ ± S.D.) and mean proportion of filled seed per cone fertilized by supplementally applied pollen, as influenced by pollen dilution, number of applications and cone-bud phenology at time of treatment

Number of Applications	Dilution[1] 100 % Filled Seed (No.)	SMP (%)	N[2]	50% Filled Seed (No.)	SMP (%)	N[2]
Single						
Early	29±9	3±6	3	22±3	15±24	4
Prime	37±2	76±5	2	32±8	50±27	14
Late	40±7	32±13	13	39±4	35±21	4
Double						
Early	29±7	50±24	6	26±8	47±6	3
Prime	43±7	53±17	8	43±13	53±9	5
Late	40±6	51±3	6	44±13	33±14	13

[1] Pollen was applied pure or diluted 1:1 with dead pollen.

[2] N is the number of cones per treatment used to calculate means and standard deviations.

Table 3: Mean seed yields and electrophoretically confirmed proportion of filled seed fertilized by SMP, on a per cone basis. Effects of main factors (numbers of applications, pollen dilution and cone-bud phenology) are shown

Factor	Total Seed Number	Filled Seed Number	Filled Seed Percent	SMP Seed (%)	N[1]
Application					
1	54.5 a	32.5 a	59.6 a	39.1 a	30
2	58.3 a	39.7 a	68.1 a	45.9 b	41
Dilution					
Pure	57.2 a	37.2 a	65.1 a	46.1 a	28
1:1	56.3 a	36.2 a	62.9 a	40.9 a	43
Phenology					
Early	53.2 a	26.7 a	49.0 a	28.8 a	16
Prime	57.0 a	37.5 a	65.4 a	54.1 b	29
Late	58.4 a	41.8 b	71.1 b	38.2 a	26
Control[2]	39.3	18.9	48.2	10.0	37

Numbers followed by different letters (within a factor) vary significantly at $p = .05$.

[1] Number of cones analyzed.

[2] A collection of 37 cones from below treatment branches, phenology unknown.

How accurate was the electrophoretic technique in detecting successful fertilizations? The results suggest it was very effective in detecting clone 76 pollen. Genetic theory holds that 75% of the seed identified as successes (probable + unambiguous) should be "unambiguous". The observed value was 74.9% ($x^2 = 1.01$, 1 d.f.). Therefore it is unlikely the other three clones within the two orchards which possessed the appropriate three locus genotype contributed appreciably to the observed success rate since only "probable" seed could have been produced by them. Had these clones contributed, the "ambiguous" value would have been considerably reduced. The probability of detecting clones from outside the orchard with the appropriate "unambiguous" genotype is vanishingly small (determined by multiplying population gene frequencies of marker alleles and the probability of a pollen grain being from outside the orchard). The most likely source of error comes from natural pollinations by clone 76 itself. There are a number of reasons why we believe this situation to be rare. There were only three pollen-bearing ramets of clone 76 in the block, the nearest being over 100 meters from the target tree. These ramets were competing with at least 350 other pollen-bearing ramets in the Coos Bay block (they represented less than 1% of the pollen source). There were no unusual circumstances favoring windborne 76 pollen. In fact, there were several other clones with large pollen crops immediately surrounding the target tree making competition more vigorous. The fact that 10% of control seed was apparently fertilized by clone 76 pollen seems disconcerting at first. However, these cones were picked primarily from branches 2 to 3 whorls below treated branches. SMP treatments are directed downward and delivered pollen was observed traveling many meters. Under more controlled conditions in a different experiment, SMP success was even observed on untreated ramets several meters distant from a target ramet.

In this study filled seed counts were about twice as high for SMP cones as for control cones on the same tree. This roughly equates with the electrophoretic measure of success. Although similar here, it should not be construed that one is as good as the other as indicators of fertilization success. Filled seed counts may be very misleading, especially in older orchards with heavy pollen production. For example, filled seed are more plentiful in late than in prime cones, but SMP success is appreciably higher in the latter. The biochemical method of detection remains the only definitive approach and should be used before making major decisions about costly operational practices like SMP. If allozyme analysis is used for SMP verification, it is preferable that pre-selected pollen parents with appropriate markers be used.

As for SMP as an operational treatment in conifer orchards, it would appear promising. Ultimately the decision to use SMP is an economic one, dependent on the balance of costs (pollen collection, extraction, storage, delivery) and benefits (improved genetic gain and seed yields). Also the larger the land base to be planted, the greater the opportunity for deployment of what may otherwise be a marginal operation. We feel confident, based partly on the results of this study and a more extensive study underway this year, that SMP will someday be a part of our orchard operations.

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ISOZYME HETEROZYGOSITY AS A SELECTION CRITERION FOR YIELD IMPROVEMENT IN DOUGLAS-FIR

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ABSTRACT

Family mean isozyme heterozygosity was correlated with volume and plot-to-plot volume variability in six to ten year old full-sib progeny tests of Douglas-fir (Pseudotsuga menziesii (Mirb.) Franco). No evidence of heterozygote superiority in growth rate or growth rate genotype x environment stability was found. However, a small, but significant negative association between growth rate and the presence of rare alleles was detected. All things considered, isozyme heterozygosity does not appear to offer much promise as a selection criterion for growth rate in Douglas-fir.

Keywords: Pseudotsuga menziesii, tree breeding, heterosis.

RÉSUMÉ

L'hétérozygosité isozymatique familiale moyenne a été corrélée avec le volume et la variabilité volumique interparcelles de demi-fratries de douglas (Pseudotsuga menziesii (Mirb.) Franco) âgées de six à dix ans. On n'a trouvé aucune preuve de supériorité des hétérozygotes dans le rythme de croissance ou dans la stabilité du génotype x environnement dans le taux de croissance. Cependant, on a détecté une association négative minime mais significative entre le taux de croissance et la présence d'allèles rares. Tout considéré, l'hétérozygosité isozymatique ne semble pas offrir beaucoup de promesses comme critère de sélection pour le taux de croissance chez le douglas.

Mots-clés: Pseudotsuga menziesii, amélioration génétique des arbres, hétérosis.

Because isozymes can be assayed inexpensively at early ages, their use for selection of economically important traits is extremely attractive. In agronomic crops there are several examples where isozymes have been successfully utilized to select for morphological and physiological traits. In tomato, nematode resistance (Rick and Fobes, 1974), cold tolerance (Vallejos and Tanksley, 1983), and fruit weight (Tanksley et al., 1981) have been improved through selection for specific isozymes; in corn, isozymes have been

employed in selection for yield and ear number (Stuber *et al.*, 1982) and might be useful in selection for oil content (Tsaftaris and Scandalios, 1983).

In all of the above examples, isozymes served as markers for genes controlling the traits of interest. The isozymes were assumed to have no direct influence on the economic trait, but specific isozyme alleles were identified which were linked with alleles confering superior plant properties (Soller and Beckman, 1983; Tanksley, 1983). The use of isozyme markers requires strong linkage disequilibrium. For inbred plant populations (such as those in the above examples), linkage disequilibrium is the rule. However, for outcrossing populations, linkage disequilibrium is small and random in the absence of joint selection (Lewontin, 1974). Hence, isozyme markers are of little utility for indirect selection in outcrossing populations (Orton, 1983) such as are found in most current tree breeding programs.

Isozyme heterozygosity has also been suggested as a criterion for selection of commercially important traits, specifically in trees (Mitton *et al.*, 1981). This is based on Lerner's (1954) Theory of Genetic Homeostasis, which predicts heterozygote superiority for fitness traits, such as growth rate and fecundity, in natural environments which are subject to temporal environmental fluctuations. Corroborative evidence includes numerous observations of heterozygote excess (including one in Douglas-fir (Shaw and Allard, 1982) and significant correlations between isozyme heterozygosity and various fitness traits in plant and animal populations (Mitton and Grant, 1984). In trees, isozyme heterozygosity has been positively correlated with growth rate for *Pinus rigida* Mill. (Ledig *et al.*, 1983), *Pinus attenuata* Lemm. (Strauss, pers. comm.) and *Populus tremuloides* Michx. (Mitton and Grant, 1980), with stability of growth rate in *Pinus contorta* Dougl. ex. Loud. (Knowles and Mitton, 1980) and with fecundity in *Pinus ponderosa* Dougl. ex. Laws. (Linhart and Mitton, 1985).

The evidence is by no means conclusive, however. Heterozygote deficiencies have been observed as often as excesses (Brown, 1979), and the correlations between heterozygosity and fitness traits are confounded by inbreeding (Ledig *et et.*, 1983) and sampling effects (Shaw and Allard, 1982) and are inconsistant (Mitton *et al.*, 1981).

In this paper we examine the relationship between isozyme heterozygosity and (1) growth rate and (2) growth variability in coastal Douglas-fir. Several measures were taken to eliminate problems encountered in previous studies. Inbreeding effects were removed by using full-sib crosses between unrelated parents. Spurious linkage disequilibrium effects were minimized through the use of several widely separated populations. Finally, family means were used to minimize environmental "noise". Under these conditions, significant correlations would suggest true heterozygote advantage and would encourage the use of isozyme heterozygosity as a criterion for selection in yield improvement.

MATERIALS AND METHODS

The Population

The isozyme data were collected from 356 phenotypically selected

Douglas-firs representing much of the low elevation range in western Washington and Oregon. From these parents 178 full-sib families were produced in a bi-parental mating scheme. Parents of a cross were from the same breeding zone, but were growing no closer than one mile apart. The progeny were reared in containers in a greenhouse and transplanted to field sites without culling. Mortality in the nursery phase was negligible. Each family was represented in the field by a four-tree, non-contiguous plot in each of 16 to 32 replicates, divided among two to four locations within the breeding zone of the parents. Mortality reduced family size by a maximum of ten percent. Spacing in the tests ranged from 1.22 X 1.22 to 2.44 X 2.44 m. At the time of measurement, six to ten years from planting, all plantations had achieved crown closure. Plantation mean heights ranged from 2.0 to 7.8 m.

Growth Variables

Two growth measures were correlated with isozyme heterozygosity: volume and plot-to-plot volume variability. Volume was calculated for individual trees from height and diameter according to methods developed by Kovats (1977). Family means, standardized to mean = 0 and variance = 1 at each test site were the volume data used in the correlations. Plot-to-plot variation in volume was calculated for each family as the standard deviation of the residuals after block effects were removed.

Isozyme Heterozygosity

Genotypes of the parents were determined from assays of the megagametophytic tissue in ten seeds per parent. Nineteen enzyme systems and 30 loci were utilizable. Twenty one of the loci were polymorphic at the 95 percent criterion.

Expected family mean heterozygosity was calculated for each locus assuming random mating of the gametes and absence of selection. Expected family mean heterozygosities for groups of loci were calculated as the average of expected heterozygosity over those loci. Expected family genotype frequencies were compared to actual genotype frequencies (by Chi-square) in each of six families to test the validity of the expected heterozygosity calculations. Actual genotype frequencies were determined from 100 embryos in each family, however only 13 of the loci could be assayed. Embryos were employed because of their availability and the negligable mortality of the progeny providing volume data.

Correlations

Both of the yield variables were correlated with expected family mean heterozygosity at individual loci, over all loci and over several subgroups of loci. The subgroups included (1) loci which were highly polymorphic (frequency of the most common allele $<$ 90 percent), (2) loci which were slightly polymorphic (frequency of most common allele $\geq$90 percent), (3) loci involved in glycolysis and the pentose shunt, (4) loci involved in nitrogen metabolism, (5) loci selected by multiple regression, and (6) loci selected by principal components analysis.

RESULTS

Variation in Family Mean Volume and Expected Heterozygosity

The use of full-sib family means has the advantage of reducing environmental "noise" in the measurement of volume, but it also reduces the number of observations and the total genetic variation (by one-half), thus diminishing the ability to detect significant genetic associations. In this study, family means were very effective in eliminating environmental variance and a large number of families were available. Therefore, the advantages of family means more than compensated for the disadvantages, and genetic relationships could be determined with great precision. The genetic portion of the variance among family means (family heritability) averaged 80 percent, compared to 12 percent for individual trees (individual tree heritability). The range in expected family mean heterozygosity over all 30 loci ranged from 0.18 to 0.43, and the range in family mean volume ranged from 60 to 144 percent of the progeny test means.

Are Genotype Frequencies Different than Expected?

Expected family mean heterozygosities were calculated on the assumptions that gametes united at random and viability selection was absent. Chi-square tests indicated that for 13 loci assayed in six families, the actual genotype frequencies were not different from the expected frequencies (Table 1). Only three of the 50 tests conducted showed significant deviations from expected frequencies at the five percent level, about what is expected by chance alone. Thus, while this is not a thorough test, the expected family heterozygosity values appear to be valid.

Table 1. Chi-square tests for viability selection

Locus	No. of tests	No. of signif. tests (alpha=0.05)
PGM	4	0
LAP2	5	0
CAT	5	0
G2D	5	0
GOT2	3	0
GOT3	3	0
IDH	3	0
6PGD	2	0
G6PD	5	0
DIA	5	1
MDH3	3	0
UGP1	3	1
UGP2	5	1
TOTAL	50	3

Associations Between Heterozygosity and Volume Variables

Expected family mean heterozygosity over all 30 loci accounted for less that 0.1 percent of the variation in family mean volume and no statistically significant association was detected. The relationship between volume variability and heterozygosity was equally weak and non-significant (Table 2).

When subsets of loci were considered (Table 2), a small, but significant, negative correlation between volume and heterozygosity at loci with slight polymorphism was detected. Volume was not related to heterozygosity at loci with high levels of polymorphism, and volume variability was not related to heterozygosity in either subset. No associations between volume or volume variability and groups of loci involved in glycolysis or nitrogen metabolism were found, either, but average heterozygosity in the loci, CAT, GDH, GOT2, MDH1 and UGP2, chosen by multiple regression, was positively related to plot-to-plot variability. Relationships derived through multiple regression must be viewed with caution until tested in other populations, however.

Table 2. Proportion of family variation in growth variables explained by expected family mean heterozygosity over groups of loci and signs ( ) of the association. Underlined values are significant at the 0.05 level

Basis	Volume	Volume variability
All loci	(-)0.001	(-)0.002
POLYMORPHISM		
High levels[1]	(+)0.002	(+)0.000
Low levels[2]	(-)0.027	(-)0.001
FUNCTION		
Glycolysis[3]	(-)0.005	(+)0.000
Nitrogen metabolism[4]	(-)0.003	(+)0.001
MULTIVARIATE ANALYSIS		
Multiple regression	(+)0.002[5]	(+)0.026[6]
Principal components	---	(-)0.005[7]

[1] Frequency of most common allele < 0.90.
[2] Frequency of most common allele > 0.90.
[3] Loci: ACO1-2 PGI1-2 IDH, 6PGD, G6PD, FUM, and MDH1-3.
[4] Loci: GDH, GOT1-3 FUM, MDH1-3.
[5] Loci: PGM and AP.
[6] Loci: CAT, GDH, GOT2, MDH1 and UGP2.
[7] Loci: CAT, GOT3, IDH and UGP3.

When isozyme heterozygosity at the 30 loci was correlated individually with volume and volume variability, only one test was significant at the five percent level. This is fewer than expected by chance alone.

DISCUSSION

With inbreeding eliminated, spurious linkage disequilibrium minimized, and environmental variation controlled, there was no evidence that heterozygosity in any individual locus or group of loci considered conferred superiority in growth or stability of growth. On the other hand, the presence of rare heterozygotes in nearly monomorphic loci was related to reduced growth rate, and average heterozygosity at five loci chosen by multiple regression was related to increased variability in growth. The negative relationship between rare heterozygotes and growth suggests that the rare alleles may be deleterious.

The absence of heterozygote advantage might be related to the youthfulness of the progeny. Shaw and Allard (1982) observed an increase in heterozygosity between seedling and adult Douglas-firs and Ledig *et al.* (1983) found that correlations between heterozygosity and growth rate increased with age in pitch pine. However, these authors note several alternative explanations for their results. Furthermore, juvenile growth rate is clearly important to the fitness of Douglas-fir and any heterozygote advantage in growth rate might be expected to be evident at early ages.

While it is still possible that heterozygote superiority may be expressed at later ages, the efficacy of heterozygosity as a selection criterion for growth rate or uniformity of growth rate appears to be weak. Rotation ages are becoming increasingly shorter -- Weyerhaeuser's projected rotation age is now 40 years -- and it is not likely that the relationship between heterozygosity and growth will improve substantially between the tenth and 40th years, especially since juvenile-mature correlations for volume are reasonably good in that period (Lambeth, 1980). With the size and diversity of the population considered in this study, this conclusion should be general for coastal Douglas-fir, however it should not be extrapolated to other species.

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GENETIC VARIATION IN A NITROGEN FIXING SHRUB, ALNUS CRISPA (AIT.) PURSH

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ABSTRACT

Leaf and bud tissues from approximately 30 individuals within each of 22 populations of Alnus crispa (Ait.) Pursh were used to characterize the population structure of this tetraploid species. Sampling of populations was conducted in Saguenay, Charlevoix and Québec regions of Québec province. Sixteen structural loci were inferred from a total of 11 enzymes. Of these, 9 were found to be polymorphic in at least one population and, on the average, 42% of the loci per population were polymorphic. Assuming a diploid model, average level of expected heterozygosity was 0,14 and average level of observed heterozygosity was 0,13, giving rise to an overall slight deficiency in heterozygotes. Apart from chance expectations, nearly all populations were in diploid Hardy-Weinberg equilibrium for the set of polymorphic loci surveyed. Heterogeneity among the populations was noted for 7 loci. The mean genetic distance was over 0,98. Overall, the among-population diversity seems to be low compared to the within-population diversity. The band patterns and the organization of the variability conform very well to a typical outcrosser diploid species.

Keywords: isozyme, population structure, actinorhizal plant, alder

RÉSUMÉ

La structure génétique des populations d'Alnus crispa (Ait.) Pursh, une espèce tétraploïde, a été étudiée dans les régions du Saguenay, de Charlevoix et de Québec dans la province de Québec. En moyenne, 30 individus par population furent échantillonnés au sein de 22 populations. L'analyse électrophorétique a été faite à partir de tissus foliaires et de bourgeons. Seize loci structuraux ont été investigués à partir de 11 systèmes enzymatiques. De ces loci, 9 se sont montrés polymorphiques dans au moins une population et en moyenne, 42% des loci par population étaient polymorphiques. Basé sur un modèle diploïde, le niveau moyen d'hétérozygosité espéré était de 0,14 et le niveau moyen d'hétérozygosité observé était de 0,13, révélant ainsi une légère déficience en hétérozygotes. La plupart des populations étaient en équilibre d'Hardy-Weinberg pour l'ensemble des loci polymorphiques étudiés, en supposant un modèle diploïde. Au niveau inter-population, une hétérogénéité

fut notée au sein de 7 loci. La distance génétique moyenne était supérieure à 0,98. Dans l'ensemble, la diversité observée entre les populations semble faible en comparaison de la diversité observée au sein même des populations. Les patrons de bandes et l'organisation de la variabilité s'accordent généralement très bien avec ceux d'une espèce typiquement diploïde à faible autofécondation.

Mots-clés: isozyme, structure des populations, plant actinorhize, aulne.

VARIATIONS DU PATRON DES PROTÉINES AU COURS DE L'ACCLIMATATION AU FROID D'ALNUS

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RÉSUMÉ

Trois espèces d'*Alnus* (*A. crispa* (Ait.) Pursh; *A. glutinosa* (L.) Gaertn.; *A. rubra* Bong.) ont été exposées à des conditions d'endurcissement au froid. Au cours de la période d'acclimatation nous observons une augmentation du degré de résistance au gel surtout accentuée chez l'aulne crispé. Parallèlement à la période d'endurcissement nous avons suivi l'évolution du patron des protéines sur gel de polyacrylamide en présence de SDS. Les changements observés sont essentiellement quantitatifs et l'abondance relative de certaines bandes polypeptidiques varie pendant la période d'acclimatation au froid. Nous discuterons des variations des polypeptides en relation avec le développement de la tolérance au gel.

ABSTRACT

Three *Alnus* species (*A. crispa* (Ait.) Pursh; *A. glutinosa* (L.) Gaertn.; *A. rubra* Bong.) were exposed to cold hardening conditions. During the acclimatization period we observed an increase of the level of cold hardiness, specially pronounced for American green alder. Concurrent with the hardening period, we followed the evolution of the protein pattern on polyacrylamide gel with SDS added. Changes observed were essentially quantitative and the relative abundance of certain polypeptidic bands varied during the cold acclimatization period. We discuss polypeptid variations in relation with the growth of frost tolerance.

EVIDENCE FOR EXTENSIVE COMMON DOMAIN OF SPOROPHYTIC AND GAMETOPHYTIC GENE EXPRESSION IN POPULUS DELTOIDES MARSH., P. NIGRA L. AND P. MAXIMOWICZII HENRY

by

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ABSTRACT

Interest in gametophyte-sporophyte relations in higher plants has been recently stimulated by the proposal that selection among haploid male gametophytes (pollen grains) might have a positive correlated effect on the sporophytic generation. A fundamental requirement of this selection model is that a portion of the genes expressed in the sporophyte also be expressed in the haploid pollen.

The objective of the present study was to further the proposal of male gametophyte selection by determining the extent of overlap in sporophytic and gametophytic genes and post-meiotic gene expression in *Populus deltoides* Marsh., *P. nigra* L. and *P. maximowiczii* Henry.

Male gametophyte (pollen) isozyme profiles of *P. deltoides*, *P. nigra* and their hybrids, and of *P. maximowiczii* were compared with those of the sporophyte (root tips). Fifteen enzyme systems (ACP, ACO, ADH, DIA, GDH, GOT, G-6-PDH, IDH, LAP, MDH, PER, PGM, 6-PGD, PGI and SDH) were resolved by starch gel electrophoresis for each species. Evidence was provided that sporophyte and gametophyte in these *Populus* species rely on a common structural gene repertoire. A total of 46 structural genes in *P. deltoides* and 45 genes each in *P. nigra* and *P. maximowiczii* were detected in both tissues (roots and pollen). In *P. deltoides*, *P. nigra*, and *P. maximowiczii* respectively 74%, 80% and 76% of the structural genes coding for these enzymes in the sporophyte were also found to be expressed by the gametophyte.

Five of the genes (IDH-2, MDH-4, MDH-6, 6-PGD-4 and PGI-2) coding for dimeric enzymes found in both roots and pollen were tested for post-meiotic expression. The data for MDH-6 was inconclusive. The other four genes tested were found to be expressed after meiosis, apparently transcribed and translated, in the haploid gametophyte.

The concept that selection for genes expressed in the gametophytic stage could have a marked correlated effect on the sporophytic generation of the *Populus* species studied, was thus supported.

RÉSUMÉ

L'intérêt envers les relations gamétophyte-sporophyte chez les plantes supérieures a été récemment stimulé par l'hypothèse selon laquelle la sélection parmi les gamétophytes haploïdes mâles (grains de pollen) pourrait avoir un effet corrélatif positif sur la production de sporophytes. Une exigence fondamentale de ce modèle de sélection est qu'une partie des gènes exprimés dans le sporophyte soit aussi exprimée chez le pollen haploïde.

La présente étude avait pour objet de pousser plus avant cette hypothèse de la sélection des gamétophytes mâles en déterminant le niveau de recouvrement des gènes sporophytiques et gamétophytiques et l'expression post-méïotique des gènes chez *Populus deltoides* Marsh., *P. nigra* L. et *P. maximowiczii* Henry.

Nous avons comparé les profils d'isozymes des gamétophytes mâles (le pollen) chez *P. deltoides*, *P. nigra* et leurs hybrides, de même que *P. maximowiczii*, avec ceux des sporophytes (extrémités des racines). Pour chaque espèce, nous avons résolu, par électrophorèse sur gel d'amidon, quinze systèmes enzymatiques (ACP, ACO, ADH, DIA, GDH, GOT, G-6-PDH, IDH, LAP, MDH, PER, PGM, 6-PGD, PGI et SDH). Nous avons pu mettre en évidence que le sporophyte et le gamétophyte de ces espèces de *Populus* dépendent d'un répertoire commun de gènes structuraux. Au total, nous avons détecté 46 gènes structuraux chez *P. deltoides* et 45 chez *P. nigra* et chez *P. maximowiczii* pour les deux tissus (racines et pollen). Chez *P. deltoides*, *P. nigra* et *P. maximowiczii*, nous avons trouvé, respectivement, que 74%, 80% et 76% des gènes structuraux codificateurs de ces enzymes sont aussi exprimés chez le gamétophyte.

Cinq des gènes codificateurs des enzymes dimères (IDH-2, MDH-4, MDH-6, 6-PGD-4 et PGI-2) ont été testés pour l'expression post-méïotique. En ce qui concerne MDH-6, les données ne sont pas concluantes. Les quatre autres gènes, apparemment transcrits et traduits dans le gamétophyte haploïde, sont exprimés après la méïose.

Le concept selon lequel une sélection effectuée au niveau des gènes exprimés au stage gamétophytique pourrait avoir un effet corrélatif marqué sur la génération sporophytique chez les espèces de *Populus*, est donc confirmé.

MATING SYSTEMS AND FERTILIZING POLLEN GENE POOLS OF POPULUS DELTOIDES MARSH. AND P. NIGRA L. CLONES LOCATED IN DIFFERENT COMPATIBLE SPECIES NEIGHBOURHOODS AS INFERRED FROM ISOZYME ANALYSIS

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ABSTRACT

The mating systems and effective fertilizing pollen gene pools were studied on two clones of each of Populus deltoides and P. nigra. One of the P. deltoides clones studied was located in a small natural stand of the same species, and the other in an arboretum with a variety of compatible Populus species and hybrids, such as P. deltoides, P. nigra, P. maximowiczii, P. x canadensis and P. x jackii. The two P. nigra clones were located in an arboretum of P. nigra specimens with several male clones of P. x canadensis in a neighbourhood plantation.

The genotypes of the maternal clones and of 50 seedlings of each open-pollinated progeny were studied by starch-gel electrophoresis of enzymes in the root tips. Seven enzyme systems (ACO, GOT, IDH, LAP, MDH, 6-PGD and PGI) were assayed and a total of 23 encoding loci were observed. The mating systems and fertilizing pollen gene pools were identified from the genotypes of maternal clones and their open pollinated progenies at all loci, with specific consideration to 9 marker allozyme loci.

The mating system of each clone varied with the allelic constituents in the effective pollen pool. The P. deltoides clone located in the natural stand had fertilizing pollen from related trees of the same species. The progeny of the other P. deltoides clone reflected pollen genes of P. deltoides and of a variety of compatible species such as P. maximowiczii, P. balsamifera and P. nigra, either from trees of pure species or from hybrids in the arboretum. Evidence was provided that this clone was outcrossing with P. deltoides x P. nigra hybrids and P. x jackii. P. nigra clones were outcrossing with diverse genotypes only of this species in the arboretum. The morphology of the seedlings was found to be concordant with the results of allozyme analysis.

The reproductive affinities of P. deltoides and P. nigra to compatible species and hybrids present in the neighbourhood, seem to affect their mating system. Allozymes were found to be an effective tool for the identification of mating systems and pollen gene pools of Populus species.

Keywords: mating system, pollen gene pools, Populus, allozymes, gel-electrophoresis

RÉSUMÉ

Nous avons étudié les systèmes de croisement et les bassins de gènes polliniques fertilisateurs de deux clones chacun de *Populus deltoides* et de *P. nigra*. Un des clones de *P. deltoides* étudié était situé dans un petit peuplement naturel de cette espèce et l'autre, dans un arboretum, avec une variété d'espèces et d'hybrides compatibles de *Populus*, tels *P. deltoides*, *P. nigra*, *P. maximowiczii*, *P. x canadensis* et *P. x jackii*. Les deux clones de *P. nigra* étaient situés dans un arboretum de *P. nigra*, au voisinage d'une plantation de plusieurs clones mâles de *P.* x *canadensis*.

Les génotypes des clones maternels et de 50 descendances issues de pollinisation libre furent étudiés par analyse électrophorétique du contenu enzymatique de leurs extrémités racinaires. Nous avons titré sept systèmes enzymatiques (ACO, GOT, IDH, LAP, MDH, 6-PGD et PGI) et avons observé 23 loci de codage. Les systèmes de croisement ainsi que les bassins géniques du pollen fertilisateur furent identifiés par les génotypes maternels et filiaux de tous les loci. Une attention particulière a été portée aux 9 loci allozymatiques marqueurs.

Le système de croisement de chaque clone varie selon les constituants alléliques du bassin de pollen efficace. Le clone de *P. deltoides* situé dans le peuplement naturel a reçu du pollen des arbres apparentés de la même espèce. La descendance de l'autre clone de *P. deltoides* reflète les gènes du pollen de l'espèce et d'une variété d'espèces compatibles comme *P. maximowiczii*, *P. balsamifera* et *P. nigra*, aussi bien d'arbres d'espèce pure que d'hybrides présents dans l'arboretum. Nous avons pu démontrer que ce clone se croise avec les hybrides *P. deltoides* x *P. nigra* et *P.* x *jackii*. Les clones de *P. nigra* ne se croisèrent qu'avec divers génotypes de la même espèce présents dans l'arboretum. Nous avons constaté que la morphologie des semis concorde avec les résultats de l'analyse allozymatique.

Les affinités reproductives de *P. deltoides* et de *P. nigra* avec les espèces et hybrides compatibles présents dans le voisinage semblent affecter leur système de croisement. Nous avons constaté que les allozymes sont des outils efficaces d'identification des systèmes de croisement et des bassins géniques de pollen chez les différentes espèces de *Populus*.

Mots-clés: système de croisement, bassins de gènes polliniques, *Populus*, allozymes, électrophorèse sur gel.

INTRODUCTION

The mating system of a species plays an important role in determining its genetic structure and the amount, distribution and maintenance of genetic variation in the population from generation to generation (Clegg 1980; Ritland 1983). Traditionally, estimates of mating systems were limited, especially in the longer lived plants due to the unavailability of suitable marker genes. The development of the gel-electrophoresis of enzymes technique has provided a powerful tool for such studies (Clegg 1980; Ritland 1983). Numerous models have been developed for estimating mating system parameters in

plant populations based on allozymes (Brown *et al.* 1975; Cheliak *et al.* 1983; summarized by Ritland 1983). In forest tree species, allozymes have provided measures of the mating systems of *Eucalyptus* spp. (Brown *et al.* 1975; Phillips and Brown 1977; Yeh *et al.* 1983), and of some conifer species (Rudin *et al.* 1974; Mitton *et al.* 1977; King *et al.* 1984; among others). These species are monoecious and the mating system of most of them have been described by the mixed mating (selfing and outcrossing) model.

Populus deltoides Marsh. and *P. nigra* L. are important in poplar breeding (Zsuffa 1975). These species are dioecious having male and female flowers on separate trees. Therefore, they are obligatory outcrossers. However, their females could receive fertilizing pollen from other compatible species and hybrids if located in the neighbourhood. *P. deltoides* and *P. nigra* females may also display pollen preference in pollen-mixture situations. Strong competition was found among *P. deltoides*, *P. nigra* and *P. maximowiczii* Henry pollen to fertilize *P. deltoides* ovules when studied by the pollen-mixture technique in controlled crosses (Rajora 1985). These findings generated interest for the study of mating systems of *P. deltoides* and *P. nigra* clones in open pollinated situations.

The objective of this study was to investigate the mating systems and fertilizing pollen gene pools of *P. deltoides* and *P. nigra* clones located in the neighbourhood of different compatible species and hybrids as inferred from isozyme analysis.

MATERIAL AND METHODS

Species and Clones

Two maternal clones each of *P. deltoides* and *P. nigra* were chosen for this study.

Both *P. deltoides* clones were previously used in a hybridization and interspecific pollen competition study (Rajora 1985). One of the *P. deltoides* clones (D17*) was located in a small natural stand of the same species at Cherry Beach, Toronto. The other *P. deltoides* clone (D32*) was located in an arboretum at the Ontario Tree Improvement and Forest Biomass Institute, Maple, Ontario (approximately 25 km north of Toronto), together with trees of a variety of compatible *Populus* species and hybrids, such as *P. maximowiczii* Henry, *P. nigra*, *P.* x *canadensis* Moench (natural *P. deltoides* x *P. nigra* hybrids), *P. deltoides* x *P. nigra* hybrids, *P.* x *jackii* Sarg. (natural *P. balsamifera* L. x *P. deltoides* hybrids). There was also a *P. deltoides* male tree located in the same arboretum, and another in a second neighbouring arboretum.

Both *P. nigra* clones (A and B) were located in an arboretum containing mainly clones of the same species at Allandale, Ontario (approximately 100 km north of Toronto). Male clones of *P.* x *canadensis* were also present in the neighbourhood.

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* Clones registered by this code at the Ontario Tree Improvement and Forest Biomass Institute, Maple, Ontario.

Gel Electrophoresis of Enzymes and Determination of Mating Systems

The genotypes of 4 maternal clones and of 50 seedlings from each of their open pollinated (OP) progenies were determined by starch gel-electrophoresis of enzymes in the root tips. Standardized procedures of starch gel-electrophoresis as described in Rajora (1985) were used. The following seven enzyme systems were assayed: aconitase (ACO, E.C. No. 4.2.1.3), glutamate oxaloacetate transaminase (GOT, E.C. No. 2.6.1.1.), isocitrate dehydrogenase (IDH, E.C. No. 1.1.1.42), leucine amino peptidase (LAP, E.C. No. 3.4.11.1), malate dehydrogenase (MDH, E.C. No. 1.1.1.37), 6-phosphogluconate dehydrogenase (6-PGD, E.C. No. 1.1.1.44) and phosphoglucose isomerase (PGI, E.C. No. 5.3.1.9).

Twigs were collected from each maternal clone and rooted as cuttings in Leach Tubes (polystyrene, Super Cell Containers of 164 cm^3 size). The rooting medium was Pro-mix (a mixture of 60% peat-moss, 20% perlite and 20% vermiculite). The distal one cm of actively growing root tips were collected for electrophoresis.

Open pollinated seeds from each maternal clone were collected and cleaned. The seeds were germinated in sterilized silica sand in petri dishes. The seedlings were then transplanted into Leach Tubes containing Pro-mix. The seedlings were grown under a 24 hour photoperiod. Root tips were collected for electrophoresis.

A total of 23 loci coding for 7 enzymes assayed, were observed. These loci were identified earlier in *P. deltoides* and *P. nigra* (Rajora 1985) clones as follows: ACO (2), GOT (4), IDH (2), LAP (2 in *P. deltoides*, and 1 in *P. nigra*), MDH (5 in *P. deltoides* and 6 in *P. nigra*), 6-PGD (4 in *P. deltoides* and 5 in *P. nigra*), and PGI (2). Main consideration was given, however, to the following marker allozyme genes identified for *P. deltoides*, *P. nigra*, *P. maximowiczii* and their interspecific hybrids (Rajora 1985): ACO-2, GOT-4, LAP-1, LAP-2, MDH-2, 6-PGD-2, 6-PGD-4, 6-PGD-5 and PGI-2.

Zymogram patterns of ACO, GOT, LAP, MDH, 6-PGD and PGI in root tips of *P. balsamifera* and/or *P.* x *jackii* were determined in a separate study (Rajora, documentation on file).

Mating systems and fertilizing pollen gene pools of *P. deltoides* and *P. nigra* clones were inferred from the genotypes of maternal clones and of their open pollinated progenies. The results were compared with visually observed seedling morphology (especially leaf morphology).

RESULTS AND DISCUSSION

P. deltoides

The mating system of *P. deltoides* clones varied with allelic constituents in the effective pollen pool. Clone D17 had fertilizing pollen from the trees of the same species only. These results would be expected as pollen trees of only *P. deltoides* were present in the effective neighbourhood. Most of the effective pollen trees seemed to have had the same genetic constitution as D17. This was reflected by the genotypes of the OP progenies at most of the structural loci observed including 6-PGD encoding loci, as shown in Fig. 1A. Only two variants were observed at 6-PGD-3 and at the other 21 loci all OP seedlings of D17 had the same genotype. These results indicated that the trees in the neighbourhood of D17 in the Cherry Beach population could be closely related. High similarity coefficients observed among four *P. deltoides* clones from this population, based on their genotypes at 36 loci in an earlier study (Rajora 1985), supported this interpretation.

The OP progenies of the other *P. deltoides* clone (D32), located in the arboretum, reflected pollen genes of *P. deltoides* and of a variety of compatible species such as *P. balsamifera*, *P. maximowiczii* and *P. nigra* (e.g., Fig. 1b), as identified by marker allozyme genes. Fifty percent of the progenies reflected pollen genes of *P. deltoides*. Thirty-three percent of the progenies reflected pollen genes of *P. maximowiczii*, *P. balsamifera* and/or other balsam poplars, while 17% reflected pollen genes of *P. nigra*. The morphology of the seedlings was found to be concordant with the results of allozymes analysis.

Some of the pollen genes of *P. deltoides*, *P. maximowiczii* and *P. nigra* fertilizing D32 may have come from the male trees of these species in the arboretum or in another arboretum. It seemed quite likely that D32 was outcrossing with *P.* x *canadensis*, *P. deltoides* x *P. nigra* hybrids and *P.* x *jackii*, and received pollen genes of *P. deltoides*, *P. balsamifera* and *P. nigra* from these hybrids. This was evident from the genotypes of some OP seedlings at 6-PGD-2, 6-PGD-4 and 6-PGD-5 (fig. 1b). 6-PGD-2 is typical of *P. nigra* and *P. maximowczii* and is not expressed in *P. deltoides*, and at 6-PGD-4 and 6-PGD-5, *P. deltoides* has alleles mutually exclusive of *P. nigra* or *P. maximowiczii* (Rajora 1985). The seedlings marked by x in Fig. 1b had pollen genes of *P. nigra* at 6-PGD-4 and 6-PGD-5 but not at 6-PGD-2. This could be possible if D32 ovules crossed with pollen produced by *P. deltoides* x *P. nigra* hybrids. The 6-PGD-2 gene seems to be located on a chromosome or an arm of a chromosome different from that where 6-PGD-4 and 6-PGD-5 genes are located in *P. nigra*. It is possible that at the time of pollen formation of *P. deltoides* x *P. nigra* hybrids, genetic exchange could have occurred between chromosome pairs of *P. deltoides* and *P. nigra* during crossing over, involving 6-PGD-2, 6-PGD-4 and 6-PGD-5 genes. This could have resulted in some pollen grains lacking the 6-PGD-2 gene of *P. nigra* and having all other 6-PGD genes of both *P. deltoides* and *P. nigra*. This would explain the observed genotypes of seedlings marked with x in Fig. 1b. These observations should be confirmed by controlled crossing experiments. The results, however, clearly indicate that D32 was crossing with *P. deltoides* x *P. nigra* hybrids.

P. balsamifera pollen genes in OP progenies of D32 possibly came from the two male trees of *P.* x *jackii* in the arboretum. There was no male tree of *P. balsamifera* in the neighbourhood. Thus the results suggest D32 was crossing with *P.* x *jackii*. A similar phenomenon at 6-PGD-2, 6-PGD-4 and 6-PGD-5, as previously discussed for some seedlings representing *P. nigra* pollen genes, was also observed in some D32 OP seedlings representing *P. balsamifera* pollen genes (Fig. 1b marked by y).

The results further suggest that *P. deltoides* prefers pollen genes of its own species. As a second choice, *P. deltoides* preferred *P. maximowiczii* and *P. balsamifera* pollen genes over *P. nigra* pollen genes. This indicates *P. deltoides*'s higher reproductive affinity to balsam poplars than to consectional *P. nigra*. The results were in good agreement with the pollen species preference of *P. deltoides* observed by Rajora (1985).

P. nigra

All the OP progenies of both *P. nigra* clones (A and B) reflected pollen genes of *P. nigra* only. This was evident from their isozyme patterns of enzyme systems assayed, including those of 6-PGD as shown in Fig. 1c. Both *P. nigra* clones were outcrossing with different genotypes of this species in the arboretum. The observed heterozygosity at four of the polymorphic loci (Table 1) supported this interpretation. However, clone B had a more heterozygous fertilizing pollen gene pool than clone A (Table 1).

Table 1 Observed heterozygosity at some polymorphic loci in OP progenies of *P. nigra* clones

Locus	Observed heterozygosity	
	P. nigra A x open	*P. nigra* B x open
IDH-2	0.031	0.087
6-PGD-4	0.342	0.422
6-PGD-5	0.404	0.434
MDH-6	0.031	0.087

There was no evidence of *P. deltoides* pollen genes from *P. x canadensis* males fertilizing *P. nigra* clones. The documented crossability relationships between *P. deltoides* and *P. nigra* indicate that *P. deltoides* x *P. nigra* crosses are very fertile (Zsuffa 1975), while the reciprocal cross has usually been a failure (Melchior and Seitz 1968). This could be a reason for the lack of *P. deltoides* pollen genes in OP progenies of *P. nigra* clones. Another reason could be the relatively low pollen cloud from more distant *P. x canadensis* trees.

CONCLUSIONS

The mating system and fertilizing pollen gene pools of *P. deltoides* and *P. nigra* clones depended upon the presence of different compatible species and hybrids in the neighbourhood, and their genetic and reproductive affinities with *P. deltoides* and *P. nigra*. Allozymes were found to be an effective tool for studying mating systems and fertilizing gene pools.

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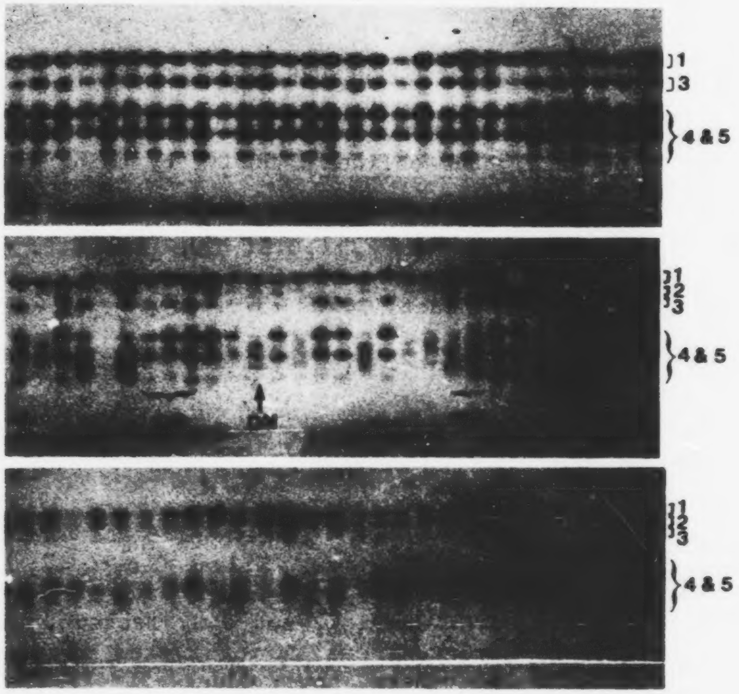

Fig. 1. Zymogram patterns of 6-PGD enzyme in the open pollinated progenies of (A) *P. deltoides* cl. D17, (B) *P. deltoides* cl. D32, and (C) *P. nigra* cl. A.

DB = *P. deltoides* x *P. balsamifera*;
DD = *P. deltoides* x *P. deltoides*;
DM = *P. deltoides* x *P. maximowiczii*;
DN = *P. deltoides* x *P. nigra*.
Encoding loci are identified by numbers.

AN OVERVIEW OF SALIX BREEDING IN ONTARIO

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ABSTRACT

The growth characteristics of some of the major North American willow species, including *Salix amygdaloides* Anderss., *S. bebbiana* Sarg., *S. discolor* Muhl., *S. eriocephala* Muhl., *S. exigua* Nutt., *S. lucida* Muhl., *S. pellita* Anderss., and *S. petiolaris* Smith have been investigated for their potential use in short rotation, biomass production systems. Interspecific hybridisation has been evaluated as a breeding method for developing superior clones and some of the crossability relationships between the above species have been identified. Artificial hybridisation under greenhouse conditions was carried out by synchronizing the flowering phenology of these different species to avoid the difficulties involved in the handling and storage of willow pollen. Some of the breeding problems encountered were poor rootability in certain species, suspected apomictic seed production and hybrid inviability.

RÉSUMÉ

Les caractéristiques de croissance de quelques espèces majeures de saule d'Amérique du Nord, y compris *Salix amygdaloides* Anderss., *S. bebbiana* Sarg., *S. discolor* Muhl., *S. eriocephala* Muhl., *S. exigua* Nutt., *S. lucida* Muhl., *S. pellita* Anderss. et *S. petiolaris* Smith ont été étudiées en vue de leur éventuel emploi dans des systèmes de production de biomasse sur de courtes rotations. On a évalué l'hybridation interspécifique comme méthode de mise au point de clones supérieurs et on a identifié certaines relations de croisement entre les espèces énumérées ci-dessus. On a mené l'hybridation artificielle dans les conditions de la serre en synchronisant la phénologie de la floraison de ces différentes espèces de manière à éviter les difficultés qu'impliquent la manutention et l'entreposage du pollen de saule. Le faible taux de racinement de certaines espèces, la production supposée de semences apomictiques et la non-viabilité des hybrides sont quelques-uns des problèmes de croisement rencontrés.

INTRODUCTION

The commercial exploitation of willows (genus *Salix*, family *Salicaceae*) has not played a significant part in North American forestry practice. The only possible exception to this has been the harvesting of *S. nigra* Marsh. stands along the lower Mississippi River Valley (Randall 1973). *Salix nigra* and several closely related species, *S. amygdaloides* Anderss., *S. caroliniana* Michx. and *S. goodingii* Ball. are the major tree-form willow species of North America but their commercial exploitation has been minimal due to poor form, absence of larger commercially viable stands and a wood quality not lending itself to major industrial uses, especially in the presence of the rich diversity of more favourable hardwood species with which this continent has been blessed. However, the advent of short rotation, intensively managed biomass plantations is resulting in a reappraisal of the potential of the genus to supply part of the world's future demand for wood fibre, energy and chemicals.

Interest in this relatively unexplored North American resource is justified on the following grounds: (1) the rapid juvenile growth and ease of establishment of many willow species using unrooted stem cuttings, (2) their adaptability to marginally-productive sites, (3) their extreme cold hardiness which makes many species ideally suited to the cold temperate and subarctic climates of Canada, (4) the potential for intensive management and mechanized harvesting, (5) the immense genetic diversity within the genus and (6) the unique opportunities for its manipulation through interspecific hybridisation and selective breeding. In this paper, some recent efforts to investigate this genetic diversity, in particular, the possibility of recombining traits of interest from several species into a single clone through interspecific hybridisation are discussed.

Since 1982, cooperation among Belgium, Canada, Finland, Ireland, Sweden and the United States, within the framework of a cooperative project (CPB-4) under the International Energy Agency's Forestry Energy Agreements, has funded field collections of the most promising North American willow species. The bulk of these collections have been propagated and maintained at the Ontario Tree Improvement and Forest Biomass Institute (OTIFBI) at Maple, Ontario, prior to redistribution to the countries participating in the project. This has provided an opportunity to assess some aspects of our major willow species, such as their physical properties, field performance, reproductive biology, protocols for artificial hybridisation, interspecific crossability relationships, genetic variation and the characteristics of F_1 interspecific hybrids in progeny trials.

CLASSIFICATION, DISTRIBUTION AND ECOLOGY

The genus *Salix* consists of over 300 species worldwide, and the majority are restricted to the northern hemisphere (Rehder 1974). Some 70 species are native to North America (Fowells 1965), although only about half of these are suitable for exploitation in short rotation biomass plantations. Species native to Ontario which have been used in breeding studies are identified in Table 1.

The genus Salix has been classified into 3 major subgenera by Skvortsov (1968) based largely on flower morphology and inferences made about the phylogenetic history of species: Salix, Vetrix and Chamaetia. However, Dorn (1976) only recognizes 2 subgenera: Salix and Vetrix. The dwarf, procumbent species have been assigned to subgenus Chamaetia by Skvortsov (1968). Only species from subgenus Salix and Vetrix are likely to be of interest for biomass production. The subgenus Salix contains most of the taller tree-sized willows predominating in the warmer temperate and subtropical climates. The subgenus Vetrix contains most of the taller shrub species most commonly found in the cool temperate zone climates. This latter group is believed to be derived from the phylogenetically more primitive subgenus Salix (Dorn 1976).

Although willows are generally associated with poorly drained wetland habitats, species such as S. bebbiana[1], S. exigua, and S. humilis are adapted to well drained sites. Most willows prefer mineral soils regardless of the texture (from gravel to clay) as opposed to soils with high organic matter contents. This large range in soil texture and moisture preferences within the genus (and in some cases within species) may be useful in breeding clones with either very specific site adaptations or clones with a high tolerance over a wide range of sites. The range of sites for which well adapted willow species can be found includes the following: (1) permanently inundated swamps, (2) seasonally inundated wetlands associated with floodplains, (3) gravel and sand plains associated with riparian habitats, (4) wet upland meadows, (5) impervious clay soils and (6) the moist but well drained coarse textured upland soils of the boreal forest zone.

COLLECTION AND PROPAGATION

Since 1982, much of the North American range of Salix, including the southeast, northeast and midwest U.S.A., and eastern and northwestern Canada, has been sampled to obtain a suitable representation of the most promising species for testing. For successful rooting, stem cuttings from branches of second and third year old wood were collected throughout the growing season. Stem cuttings measuring about 25 cm in length and 8 to 12 mm in diameter were sealed in plastic bags, and stored in styrofoam coolers with ice for shipment. For propagation, cuttings were sectioned to 12 cm in length and rooted in a soil medium of 2 parts sand to 1 part commercial peat mixture (PROMIX-B).

A greenhouse environment was not required for rooting of clones but the subsequent growth of new shoots from northern species can only be maintained by extended photoperiods using supplemented light. When northern species adapted to long days were outplanted at OTIFBI, many of them entered dormancy by the end of June. The roots of clones from as far south as Florida survived the winter of 1984/85 in field plantings and produced coppice shoots during the 1985 growing season. However, it was not possible to assess winter damage to above ground biomass since their stems were harvested for redistribution.

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[1] Authorities for systematics indicated in Table 1.

Table 1. Salix species used in artificial hybridisation studies

Taxonomical Classification			Habit and Usual Height (m)	Botanical Range	Chromosome[1] Number (2n)
Subgenus	Section	Species			
Salix	Humboldtianae	S. amygdaloides Anderss.	tree - 17	N. central USA	38
	Salicaster	S. lucida Muhl.	shrub - 6	Eastern N. America	76
	Longifoliae	S. exigua Nutt.	shrub - 5	Across USA/Canada	38
Vetrix	Cordatae	S. eriocephala Muhl.	shrub - 4	Boreal N. America	38
	Vetrix	S. bebbiana Sarg.	shrub - 6	Across Canada	38
		S. discolor Muhl.	shrub - 7	E. central USA/Canada	76
		S. petiolaris Smith	shrub - 7	Canada/Northern USA	38
	Vimen	S. pellita Anderss.	shrub - 4	Eastern Canada	76

[1] Suda and Argus, 1968

Flower branches were collected from locally available species to initiate a breeding program aimed at establishing the crossability relationships among some of the major North American species and to develop a methodology for further breeding studies.

BREEDING STUDIES

The dormant branches of the female plants of most species root simultaneously with the production of flowers. Branches from male plants can be forced as potted stock or simply by placing in water. The oily nature of willow pollen complicates its handling and storage, and it may be preferable to synchronize the flowering so that fresh pollen is continuously available as female flowers enter receptivity. In order to carry out such a synchronization for interspecific hybridisation, a knowledge of the phenological characteristics of the various species is important. The 8 species currently being studied at OTIFBI occur sympatrically and are not always spatially separated in nature. Interspecific selection pressures may have enforced partial reproductive isolation between these species through phenological differentiation.

Considerable variation has also been found between different species studied in the length of the receptive period of female flowers. This period varied from a maximum of 48 hours in most clones of S. petiolaris to a receptive period of more than 6 days in S. lucida and S. amygdaloides. Generally, the precocious flowering species (those flowering before the onset of vegetative growth) have reduced periods of flower receptivity in comparison with late flowering species. Differences in the length of the receptive period may reflect natural selection for reproductive isolation under the pressure of interspecific pollen competition arising from the sympatric association of so many closely related species. From a more pratical viewpoint, the willow breeder must be aware of these differences between species because they affect seed set results in artificial pollination studies.

Under normal greenhouse conditions (20 to 25°C and a 16-hour photoperiod supplemented with artificial light), seed matures within 3 to 4 weeks for all the species studied. Seed germination on moist silica sand in petri dishes occurred over a 3 to 4 day period, and the germination rate for all species was normally above 90 percent. Seed set per flower capsule varies from an average low of 3 seeds per capsule in S. petiolaris to an average above 20 seeds per capsule in S. lucida and S. exigua. Over 1500 seeds were counted from a single catkin of S. eriocephala. Within one week from germination in petri dishes, the young germinants were transferred to larger containers for subsequent growth under greenhouse conditions. Seedlings were normally large enough for field planting after 3 months in a greenhouse. Since willows have an indeterminate growth habit, they can be outplanted in mid August and double their size by October. After one full growing season in the field, flower branches can be collected from seedlings for artificial pollination studies.

Among the minor breeding problems encountered with willow, were poor rootability and possible apomictic seed production in some species. *S. discolor* and *S. bebbiana* were among the difficult-to-root species, and should be rooted one year prior to any planned pollination studies. The rooted cuttings should then be transferred to larger containers and allowed to develop to their full extent, entering dormancy under natural conditions. Such plants should produce flowers the following growing season.

Certain types of apomictic seed production, possibly pseudogamy, may interfere with artificial pollination attempts, and have been suspected in certain female clones of *P. petiolaris* and *S. eriocephala*. Seedling progeny raised from controlled crosses with certain clones of *S. petiolaris* have resulted in arrays of female seedlings characterized by a clone-like uniformity in terms of their growth characteristics and their morphological and phenological traits. These families produced no male plants which is unusual for dioecious species that normally produce the expected 1:1 ratio of male to female plants. An attempt will be made to verify the possible apomictic origin of these seedlings by comparing their isoenzyme profiles with those of their male and female parents.

The putative interspecific hybrids are presently being verified using the techniques of numerical taxonomy and the analysis of isoenzyme profiles. Families representing these crosses have been outplanted in progeny tests at OTIFBI. The success of these interspecific crosses ranges from generally very poor as in the case of most *S. exigua* x *petiolaris* or *S. exigua* x *eriocephala* crosses which have produced a high proportion of inviable seedlings, to highly successful crosses such as those made between *S. pellita* x *discolor* and *S. bebbiana* x *S. petiolaris*. Despite the high proportion of inviable offspring produced by certain crosses, a small but significant proportion of successful individuals and sometimes exceptional clones have been obtained from crosses which generally show a poor F_1 viability. In view of the disruption of well adapted gene complexes, resulting from any kind of interspecific hybridisation, it is rather surprising to find such hybrid vigour.

Some interspecific crosses which appeared most promising on theoretical grounds have been unsuccessful. The incompatibility of certain interspecific crosses has been investigated using fluorescence microscopy to evaluate the role of pollen-pistil incongruity (Hogenboom 1973) in the prevention of successful fertilisation. Preliminary observations on interspecific crosses between *S. exigua*, *S. amygdaloides* and *S. lucida* have revealed a variety of inhibitory interactions including the inhibition of pollen germination on the stigma surface, the inability of pollen tubes to penetrate the stylar tissues and the failure of pollen tubes to develop properly within the style. An attempt will be made to quantify these pollen-pistil interactions through an analysis of variance.

In crosses between *S. lucida* and *S. amygdaloides* for instance, pollen seems to develop and grow quite normally within the female tissues of the foreign species and fertilisation apparently takes place. However, when *S. amygdaloides* is used as the female parent, the zygote/embryo aborts during embryogenesis and no mature seed is produced, although the flower capsules themselves develop to maturity. In the reciprocal cross (*S. lucida* x amygdaloides), mature seed often develops and shows a normally high germination rate but the young germinants usually die en masse within 2 weeks following germination. The cotyledons blacken suddenly as if attacked by a pathogen, but preliminary attempts to isolate a causal agent which can consistently be associated with this blackening have failed. Such severe instances of incompatibility or inviability will likely be difficult to overcome.

BIOMASS IDEOTYPE

Dickmann (1975) first used the concept of plant ideotype (borrowed from agronomy), to describe the ideal qualities of tree species for use in intensively managed, short-rotation biomass production systems. Among the more important traits of the ideotype shared by most willow species from the subgenera Salix and Vetrix, are the following: rapid juvenile growth, indeterminate shoot growth, upright excurrent habit, easy establishment and regeneration, acceptable wood properties and freedom from major pest problems. Zsuffa and Papadopol (1984) have refined the concept of the ideotype for a specific application to the family Salicaceae. The definition of an ideotype is useful in setting a breeding strategy. This is especially important for the genus Salix, where the immense genetic variation in terms of numbers of species available for breeding and the possibilities for interspecific hybridisation create some unique opportunities for recombining many desirable traits within a single clone in a relatively short period of time.

Although most of the Salix species studied within the frames of this project approach the ideotypes defined, many of these species also have their own particular shortfalls, whether it be (1) poor rootability, (2) excessive numbers of shoots per coppice, (3) excessive production of lateral branches, or (4) particular disease and pest problems. Other aspects related to the ideotype that might be favourably altered through interspecific hybridisation are various site and climatic tolerances, biomass volume production and properties affecting wood quality. The phenotypic variation found within species suggests that many of the above characteristics may be influenced by selective breeding within species. Intraspecific selection and breeding may be most important in the improvement of species like S. lucida and S. eriocephala which most closely approach the biomass ideotype among the species currently being studied. The present discussion will focus on observations among interspecific hybrids.

Salix discolor has one of the best upright, branchfree, coppice forms among the willows, but is one of the few species which has poor rootability. S. pellita has a poor form but roots well. Hybrids of S. pellita x discolor maintain many of the coppice form qualities and also show improved rootability. Both species are tetraploids ($2n=76$) and all crosses between them have produced relatively uniform, vigorous F_1 progeny.

Hybrids of S. eriocephala x discolor have shown much more variable results in both growth vigour and form. These progeny generally have not been as vigorous as crosses within S. eriocephala but some individual hybrids show compensatory improvement in form, as the excessively high number of shoots per stool, characteristic of S. eriocephala, is reduced.

S. bebbiana is an extremely cold hardy species which does well under subarctic conditions and in drier habitats but it has poor form, poor rootability and is not exceptionally vigorous. S. petiolaris has a better coppice form but lacks the vigour characteristic of most temperate zone willows and is adapted to very wet sites. Hybrids between these 2 species have shown a dramatically increased vigour in their first year of growth, a much improved form over both of the parental species, and represent one of the best examples of hybrid vigour arising from interspecific hybridisation.

Salix exigua has a poor coppice form, diverting too much biomass into lateral branches and root suckers but is often exceptionally vigorous and has excellent rootability. Salix petiolaris does not root as well as it should for the most economical establishment of biomass plantations. The hybrid families of S. exigua x petiolaris produce high proportions of inviable seedlings along with a few remarkably vigorous exceptions. These superior individuals have shown good form and fast growth. S. exigua is also the only willow species known to reproduce via root suckering. Results to date indicate that this trait is not inherited by the F_1 interspecific hybrids in which either S. petiolaris or S. eriocephala has been used as the male parent.

Neither S. amygdaloides (one of only 2 tree-form willows native to Canada), nor S. lucida has been successfully crossed with any other species being studied. Although closely related phylogenetically, all attempts to cross these 2 species with one another have also failed due to zygote and seedling inviability, depending on which species is used as the female parent.

SUMMARY

Some of the crossability relationships among the major North American willow species have been identified and the viability of the F_1 hybrids has been evaluated. Microscopical studies on the fate of foreign pollen in female flowers have revealed some instances of pollen-pistil incongruity, whereas other crosses, such as those between S. lucida and S. amygdaloides, did not reveal any apparent incongruity. Crosses between these 2 species appeared to result in fertilisation followed either by zygote/embryo abortion or seedling inviability depending on which species was used as the female parent. Crosses yielding viable seed resulted in varying degrees of developmental inviability, both while under greenhouse culture and after seedlings were field planted. Despite such problems of seedling inviability, some crosses between S. exigua and either S. eriocephala or S. petiolaris, produced some exceptionally vigorous progeny. Although many interspecific hybrids have shown reduced fertility, there appeared to be enough fertility to carry out advanced generation breeding.

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GROWTH AND ECTOMYCORRHIZAL DEVELOPMENT OF LOBLOLLY PINE PROGENIES INOCULATED WITH THREE ISOLATES OF PISOLITHUS TINCTORIUS

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ABSTRACT

Loblolly pine (Pinus taeda L.) progenies from controlled crosses were inoculated with different isolates of the fungal symbiont Pisolithus tinctorius. Seedlings showed genetic variation in ectomycorrhizal development, shoot height, and component dry weights. Fast growing progenies exhibited superior mycorrhizal colonization and a positive correlation between infection and total dry weight. Both fungal and host genotype influence mycorrhizal symbiosis and subsequent seedling growth.

Keywords: Pinus taeda, Pisolithus tinctorius, mycorrhizae.

RÉSUMÉ

Des descendances de pin à l'encens (Pinus taeda L.) issues de croisements dirigés ont été inoculées avec différents isolats du symbionte fongique Pisolithus tinctorius. Les semis ont démontré une variation génétique dans leur développement ecto-mycorhizien, la hauteur de pousse et les poids anhydres des composants. Les descendances à croissance rapide ont montré une colonisation mycorhyzienne supérieure et une corrélation positive entre l'infection et le poids anhydre total. Les génotypes aussi bien du fonge que de l'hôte influencent la symbiose mycorhizienne et la croissance subséquente du semis.

Mots-clés: Pinus taeda, Pisolithus tinctorius, mycorhizes.

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INTRODUCTION

Researchers have examined variation in ectomycorrhizal formation and early growth of half-sib progenies of *Pinus* spp. inoculated with different fungal symbionts. Marx and Bryan (1971) observed that slash pine (*Pinus elliottii* var. *elliottii* Engelm.) regulates the degree of ectomycorrhizal development by *Pisolithus tinctorius* (Pers.) Coker and Couch, as well as the early shoot growth following ectomycorrhizal colonization. Host genotype of loblolly pine (*Pinus taeda* L.) also influenced ectomycorrhizal formation by a single isolate of *Pisolithus tinctorius* (Long, 1973). In contrast to earlier studies, Cline and Reid (1982) observed that the benefit received by different provenances of lodgepole pine (*P. contorta* var. *latifolia* Engelm.) and ponderosa pine (*P. ponderosa* var. *scopulorum* Engelm.) following ectomycorrhizal colonization was influenced by the amount of ectomycorrhizal infection rather than host genotype. However, provenance genotype did indirectly affect seedling growth by influencing ectomycorrhizal colonization of the root system.

Few studies have examined the role of fungal genotype in the ectomycorrhizal infection process or subsequent growth response to colonization. Laiho (1970) reported that isolates of *Paxillus involutus* Batsch ex Fr. differ in their ability to form ectomycorrhizae with various tree species. Isolates of *Pisolithus tinctorius* also vary widely in ability to infect and colonize root systems of loblolly pine seedlings from open-pollinated seed sources (Marx *et al.*, 1982). These studies suggest that fungal genotype does influence ectomycorrhizal development and early seedling growth. However, the complex relationship between host genotype and fungal genotype has not been evaluated.

The purpose of this study was to evaluate: 1) the ectomycorrhizal development of three progenies of loblolly pine inoculated with three isolates of *Pisolithus tinctorius* and 2) morphological characteristics of seedlings following ectomycorrhizal colonization.

MATERIALS AND METHODS

Fungal isolates and inoculum preparation

Three isolates of *Pisolithus tinctorius* were used (Table 1). These isolates represent a range of ability to infect loblolly pine and impart a growth response. Vegetative mycelial inoculum for each isolate was grown in two-liter glass jars using procedures described by Marx and Bryan (1971).

Table 1. Sources and date of isolation of three isolates of *Pisolithus tinctorius* used to inoculate container-grown loblolly pine

Isolate no.	Source and isolation date
29	*Quercus* - *Pinus*, Georgia, USA, isolated in 1959 by B. Zak from sporocarp tissue.
145	*Quercus acutissima*, Kentucky, USA, isolated in 1974 by D. Marx from sporocarp tissue.
270	*Pinus taeda*, Georgia, USA, isolated 1980 by D. Marx from sporocarp tissue.

Seed source and seedling culture

Half-sib seed were collected from three loblolly pine selections currently under evaluation by the Department of Forestry at Oklahoma State University. Seed were stratified and stored using standard procedures. The three selections were chosen based on their BV-20 value. A BV-20 value is an estimate of a selection's breeding value, in a percent form, at age 20 (personal communication, Mr. Thomas Byram, Western Gulf Forest Tree Improvement Cooperative, College Station, TX, USA). These values state the relative superiority of selections more clearly than simple percent superiority values and allow for comparison of selections across test sites. The baseline BV-20 value of the genetic test sites under evaluation is considered to be 100; this can be viewed as the plantation average.

As can be seen from Table 2, selection 81, with a BV-20 value of 122, is considerably better than the test plantation average, and has been incorporated into second generation orchards. Selection 90 is slightly below average with a BV-20 of 95, and is being maintained in first generation orchards. Finally, selection 73, with a BV-20 of only 86, has been rogued from the program.

Table 2. Breeding values at age 20 (BV-20) of the selections under investigation. Baseline value is 100; check selections possessed a BV-20 of 92

Selection	BV-20 Value
81 (superior)	122
90 (average)	95
73 (inferior)	86

A 1:1 sphagnum moss-vermiculite mixture, fumigated with methyl bromide for 48 h, was the potting substrate (Dixon *et al.*, 1984). Mycorrhizal treatments were implemented by mixing one part inoculum with 20 parts sterile potting substrate. Noninoculated seedlings from each selection were grown in sterile sphagnum moss-vermiculite. Four-cavity (500 cc capacity) rootrainers were filled with the appropriate medium and pre-germinated seed were planted one per cavity. The rootrainers were arranged in a completely randomized block design. Each fungal x half sib family treatment combination (4 x 3) was replicated in five blocks. Seedlings were grown in a glasshouse for 20 weeks from May through September, 1982. Plants were watered on alternate days and received 75 ml of full-strength Hoagland's solution on a weekly basis.

Seedling analysis

After 20 weeks, 16 seedlings per treatment per block were harvested. Root systems were visually examined for ectomycorrhizal colonization by each test fungus. Percent ectomycorrhizal colonization was determined by methods described by Dixon *et al.* (1984). Ovendry (80°C, 24 h) weights of roots, shoots and needles were recorded. Shoot length and root collar diameter of each seedling were also measured. All data were subjected to analysis of variance and, where appropriate, differences among treatment means were compared with the least significant difference test (LSD) ($P \leq 0.05$).

RESULTS

Ectomycorrhizal infection

All isolates tested formed ectomycorrhizae with loblolly pine (Table 3). However, each progeny differed in their ability to form ectomycorrhizae with isolates of *Pisolithus tinctorius*. Generally, progeny 81 formed the most ectomycorrhizae, followed by progeny 90 and 73. Control seedlings were not contaminated with ectomycorrhizae.

Inoculation with isolate 270 resulted in abundant coralloid and bifurcate ectomycorrhizae yellow to gold in color. The Hartig net developed to the endodermis and mantles were 30 to 50 μ thick. Approximately 50 percent of seedling laterals were infected with *P. tinctorius* ectomycorrhizae 12 weeks after inoculation with isolate 270. Sporocarps of *P. tinctorius* developed in the containers of seedlings inoculated with isolate 270 in the eighteenth week of the study.

Inoculation with isolate 145 resulted in significantly less ectomycorrhizae than observed for isolate 270. Ectomycorrhizae of isolate 145 was yellow in color and Hartig net and mantle development on short roots was incomplete. When present, mantle thickness ranged from 10 to 20 μ.

Significantly fewer laterals were infected with isolate 29 for all three progenies. Ectomycorrhizae were golden brown and mantle development was visible on only a few short roots.

Table 3. Shoot length, root collar diameter, and percent ectomycorrhizal laterals of three loblolly pine progenies inoculated with four ectomycorrhizal fungal treatments at 20 weeks

Progeny	Fungal isolate	Shoot length (cm)	Root collar diameter (cm)	Total dry weight (g)	Ectomycorrhizal laterals (%)
81	control	13.2cde	4.4cd	4.1bc	0a
	29	15.1ef	4.1bc	4.4bc	18b
	145	14.3de	4.0b	4.0bc	51c
	270	17.9f	5.3e	6.6d	86e
90	control	12.0bc	4.2bc	4.3bc	0a
	29	13.0cd	4.6d	4.4bc	23b
	145	13.2cde	4.1b	5.2cd	42c
	270	14.7de	4.5cd	5.1cd	71d
73	control	9.9ab	3.2a	2.3a	0a
	29	10.4ab	3.6a	3.1ab	16b
	145	8.9a	4.0b	3.4ab	45c
	270	13.4cde	4.3bcd	4.4bc	56cd
Main Effects					
Fungi		*	*	*	*
Progeny		*	*	*	*
Fungi x Progeny		*	ns	ns	ns

[1] Means within a column not sharing a common letter differ significantly ($P \leq 0.05$) by LSD test.

[2] Significantly different ($P \leq 0.05$).

Shoot length and root collar diameter

Shoot length and root collar diameter were greatest for progeny 81, followed by 90 and 73, respectively (Table 3). Inoculation of progeny 81 with isolate 270 stimulated a 36 percent increase in height over noninoculated control seedlings. Generally, inoculation with isolates 29 and 145 did not impart a height growth response in progeny 90 and 73. Root collar diameter was significantly stimulated following inoculation of progeny 81 and 73 with isolate 270. Inoculation of family 73 with isolate 270 yielded the same results as noninoculated progeny of family 81.

Total dry weight

Total dry weight of progenies 81 and 73 were significantly increased following inoculation with isolate 270 (Table 3). Inoculation with isolate 270 resulted in a 61 percent increase in dry weight over control seedlings in progeny 81. Significant, but smaller, increases in dry weight were observed following inoculation of progeny 73 with isolate 270. Generally, inoculation with isolate 29 did not result in dry weight gains. Again, inoculation of family 73 with isolate 270 yielded results not significantly different from noninoculated family 81.

DISCUSSION

Ectomycorrhizal development was greatest on seedlings inoculated with P. tinctorius isolate 270 and least with isolates 29 and 145. Although original host and age in culture may significantly reduce virulence, isolates 19 and 145 grew rapidly in pure culture. Differential formation of ectomycorrhizae by different isolates of P. tinctorius on the same progeny suggests that fungal genotype influences ectomycorrhizal formation. Our results agree with previous studies which indicate that a single strain of fungus may infect a range of host genotypes (Laiho, 1970).

The degree of ectomycorrhiza formation on progeny 73 was low, while formation on progeny 81 was relatively high in all three progenies. Root systems of seedlings (e.g., number of laterals and short roots) harvested from each progeny were similar. Thus, the potential sites for ectomycorrhizal infection were similar in both progenies. Other factors such as host susceptibility to infection, phenology of short root formation, fungal specificity, or rhizosphere compatibility of the host and fungi may have influenced mycorrhizal formation (Harley and Smith, 1983). Differential ectomycorrhizae formation by some strains of P. tinctorius on different progenies suggests the influence of host genotype on ectomycorrhizal formation is significant. Previous studies have shown that the potential for mycorrhiza formation was highly dependent on seed source (Moser, 1958; Wright and Ching, 1962; Lundeberg, 1968; Marx and Bryan, 1971).

Growth of all progenies was significantly increased following inoculation with P. tinctorius isolates 145 and 270. This growth response was generally found when ectomycorrhizal infection was 50 percent or greater. These results suggest that a minimal level of root system colonization is necessary for a virulent symbiont to impart a growth response. Marx et al. (1982) also concluded that a threshold infection level of approximately 50 percent was necessary to improve the growth of southern pines.

The superior growth response of seedlings inoculated with isolates 145 and 270 and relatively poor growth of seedlings inoculated with isolate 29 suggest differential ability of strains to benefit seedling growth. Several workers have demonstrated that ectomycorrhizal strains within a species differ in element uptake efficiency, adaption to water stress, plant growth regulator production, and host carbon consumption (Harley and Smith, 1983). Because the seedlings in this study were grown in a uniform environment and received equal amounts of fertilizer, water, and light, strain superiority in nutrition or water uptake may be discounted. Other factors such as substantial host carbon consumption by isolate 29 may have resulted in the large seedling growth differences (Bevege et al., 1975).

Growth responses due to ectomycorrhizal formation on loblolly pine were strongly influenced by host genotype. Superior growth of hosts, such as progeny 81, remained superior regardless of fungal partner. Long (1973) and Marx and Bryan (1971) also suggested that pine genotype controls the benefit seedlings obtained from ectomycorrhizal relationships. In contrast, Reid and Cline (1982) concluded that the benefit received by lodgepole and ponderosa pine was directly controlled by the amount of mycorrhizal colonization rather than the host genotype. Host genotype indirectly influences seedling growth by controlling the rate and degree of mycorrhizal colonization.

In all seedling variables measured, inoculation of an inferior host family with an ectomycorrhizal fungus resulted in growth comparable to a noninoculated superior host family. While we do not propose that inoculating trees with mycorrhizal fungi supersedes the need for forest tree improvement, we do suggest that mycorrhizae research should be integrated into tree improvement programs. Such consideration could result in retaining so-called borderline genotypes within a breeding program which would in turn maintain a broader genetic base for advanced generation breeding.

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PROSPECTS FOR GENETIC MANIPULATION OF WOOD QUALITY

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ABSTRACT

Wood specific gravity and lignin content are contrasted as alternative approaches to manipulating wood quality. Despite little research having been done on optimizing the lignin content of xylem prosenchyma (tracheids, fibers and vessels) in tree stems, there is evidence that the cambial genome can be manipulated to this end, either through selection and tree breeding or by genetic engineering.

RÉSUMÉ

Le poids spécifique et le contenu en lignine du bois sont mis en opposition comme approches alternatives pour manipuler la qualité du bois. Bien qu'on ait fait que peu de recherche sur l'optimisation du contenu en lignine des prosenchymes du xylem (trachéïdes, fibres et vaisseaux) dans les tiges, il existe des preuves que l'on peut à cette fin manipuler le génome du cambium, par la sélection et le croisement dirigé des arbres ou par génie génétique.

INTRODUCTION

The theme of this 'predictive' paper is that substantial opportunity exists to optimize the quantity of lignin produced by trees during wood formation. The rationale for this is both economic and practical. Lignin decreases pulp and paper yields and is costly to extract. Neither excessively or poorly lignified structural woods are of much value.

The enzymology of lignification is well understood (Higuchi 1985) and consequently there can be little doubt that the capacity for lignification is a highly heritable trait. In addition to approaches used in traditional tree improvement programs, the manipulation of lignification genes may well be possible through test-tube genetic engineering.

These ideas are supported below, but first I attempt to place wood-quality improvement into wider perspective by considering the significance of specific gravity to wood quality.

MANIPULATING WOOD QUALITY BY ALTERING WOOD SPECIFIC GRAVITY - A CRITIQUE

Most tree improvement programs assess wood specific gravity as the primary means to improve wood quality (Zobel and Talbert 1984) because wood strength, as determined with samples from 'wild' woods, is linearly related to specific gravity (Panshin and de Zeeuw 1980). Furthermore, specific gravity is not difficult to measure and is a trait of moderate to high heritability (Zobel and Talbert 1984).

The main components of wood substance are cellulose, hemicelluloses and lignin. These have specific gravities of 1.6, 1.6 and 1.3, respectively (Stamm and Sanders 1966; Jones 1971). Wood substance of both hardwoods and conifers, whether earlywood or latewood, hovers around 1.5 specific gravity (Kellogg and Wangaard 1969).

If all voids were eliminated from wood, the maximum possible specific gravity would be approximately 1.5, a hypothetical wood, of course, because it would not meet the transpiration requirement of the crown. Wood specific gravities range between 0.3-0.5 for temperate-zone conifers and 0.3-0.8 for hardwoods (Panshin and de Zeeuw 1980). The spaces occupied by lumens, perforations, pits and cell-wall pores offset the contributions made by cell-wall materials.

At the tissue level, wood consists of annual layers of prosenchyma and parenchyma, usually subdivided into earlywood and latewood on the basis of arbitrary criteria. Latewood commonly has prosenchyma with lesser radial diameters, thicker secondary walls, and fewer bordered pits than that of earlywood. Consequently, latewood exceeds earlywood in specific gravity. Parenchyma exhibit little variation from earlywood to latewood. The influence of parenchyma on wood specific gravity is poorly researched, although it is well known that parenchyma content of wood can vary substantially (Savidge 1985).

From this analysis, it is evident that attainment of increased wood specific gravity in trees is no guarantee of improved wood quality. Hopefully the specific gravity increases already obtained in tree improvement programs can be attributed to increased latewood formation, or to a more uniformly thick-walled prosenchyma content across growth layers, without concomitant negative effects on hydraulic conductivity. It is also possible that specific gravity increases are occurring in association with a reduction in lignin content with little change in cell-wall thickness; for example weakly lignified bark can yield higher specific gravity values than those of wood (Martin 1969). I would recommend therefore that tree improvement programs focussing on wood quality support their specific-gravity determinations with anatomical and chemical analyses.

NATURAL VARIABILITY IN THE LIGNIN CONTENT OF WOOD

Variability in lignin content (and composition) within and between xylem cells and across xylem layers has been described (Panshin and de Zeeuw 1980; Obst 1982; Timell 1982; Whiting and Goring 1982). The lignin content of normal conifer woods approximates 30%, while that of hardwoods is nearer 20%. In conifer tracheids, well over 50% of lignin occurs in the S_2 layer where lignin concentration is lowest but cell-wall volume is greatest. The highest concentration of lignin, amounting to 10% of total lignin, is present in the region of thin, compound, middle lamella.

Lignin content appears to vary quite independently of specific gravity. Lignin is higher in earlywood than in latewood and lignin varies little along the length of the stem. Specific gravity is highest in latewood and decreases axially with distance from the tree base. Genotypes having either high or low specific gravity have yielded similar lignin contents (Hiett _et_ _al._ 1960; van Buijtenen _et_ _al._ 1968).

There is little research on variability in lignin content between clones. Nilsson (1943) found lignin content between aspen (no scientific name given) clones to vary between 16-19%. Basbous (1950) and Jayme and Harder-Steinhäuser (1954) indicated possibilities for improving lignin content of hybrid _Populus_. Hiett _et_ _al._ (1960) and van Buijtenen _et_ _al._ (1968) found little difference in lignin content between clones of _Pinus_ _elliotti_ and _P._ _taeda_, respectively; however, in these studies few trees were used. I know of no studies on lignin content of wood obtained from progeny tests.

Differences in lignin content among wild trees and between provenances clearly exist. Nilsson (1943) noted substantial, statistically significant differences in lignin content (26-31%) between 148 spruce (scientific name not given) trees, 70 years of age. Van Buijtenen _et_ _al._ (1961) reported lignin variation from 27 to 30% in wood of 24 even-aged, wild _Pinus_ _teada_. Lommatzsch (1940), investigating "spruce wood" from Saxony, Norway and Latvia, found German-grown wood to have the highest and the Norwegian wood to have the lowest lignin content. Prosinski _et_ _al._ (1954) studied variously aged, mature _Picea_ _abies_ from four regions of Poland, reporting northern trees to contain more lignin than southern trees. Schütt (1958) found the lignin content of _Pinus_ _contorta_ from both Washington and Oregon to be several per cent less than that in trees from Kamloops, British Columbia.

LIGNIFICATION AS INFLUENCED BY ENVIRONMENT AND GENETICS

There seems to be a consensus, albeit based on limited evidence, that cambial derivatives have the complete set of enzymes needed to take phloem-derived sugars into the shikimate pathway to phenylalanine and on to lignin monomers. On the other hand, Phillips (1954) reported that lignification in developing xylem of _Fraxinus_ could be markedly reduced by exclusion of light from foliage, and considerable additional evidence supports the concept that light regulates lignification (Ishakawa and Takaichi 1957; Grand _et_ _al._ 1982). Because root wood lignifies in the dark, it is probable that light affects lignification by influencing production in leaves of mobile regulatory substances, or possibly by promoting transpiration. All available evidence suggests that high auxin levels promote lignification, possibly in concert with ethylene and cytokinins, while gibberellins and abscisic acid inhibit it (Savidge and Wareing 1981; Savidge 1985).

Little is known of the effects of photoperiod, temperature, water stress, or air quality on lignification. There is limited evidence that calcium, zinc and boron directly influence lignification enzymes but much more research is needed with these and other nutrients before the picture will be clear.

Lignification mutants have been identified among monocots (Grand et al. 1982) but whether these are due to altered hormonal or cambial biochemistry remains to be determined.

LIGNIN AND POLYSACCHARIDE PRODUCTION ARE SEPARATELY REGULATED

Empirical observations suggest that there exists weak, if any, coupling between genes underlying production of cell-wall polysaccharides and those coding for lignin. For example, it is well established that primary walls can lignify without associated deposition of secondary-wall lamellae (Vance et al. 1980). On the other hand, secondary-wall lamellae can consist almost entirely of polysaccharide; e.g. the gelatinous layer of tension-wood fibers consists almost entirely of cellulose. Lignin deposition follows rather than accompanies polysaccharide deposition during differentiation of normalwood tracheids. Compressionwood contrasts markedly with normalwood in having low extracellular lignin and twice the quantity of lignin in its S_1 and S_2 layers (Timell 1982).

That cambial derivatives are not committed to produce both polysaccharide and lignin during prosenchyma development can also be supported with experimental data. In conifers, high concentrations of exogenous auxin promote compressionwood development, and in hardwoods low auxin levels are associated with tensionwood development (Savidge and Wareing 1981). In *Fraxinus*, in addition to the light-promoted lignification described above, exogenous gibberellic acid eliminated phloroglucinol-HCl detectable lignin, but not Mäule-reaction lignin, from otherwise normal vessel elements, fibers and tracheids (R.A. Savidge, unpublished). Grand et al. (1985) used organic inhibitors of cinnamyl-alcohol dehydrogenase, an enzyme associated specifically with lignification, to reduce lignin synthesis in poplar stems. Smart and Amrhein (1985) used a specific inhibitor of phenylalanine-ammonia lyase to block lignification in *Vigna*; these workers observed secondary-wall polysaccharide deposition and pit development to proceed normally in developing xylem despite the complete absence of lignin.

CONCLUSIONS

Despite previous recognition that trees might be selected for their lignin content (e.g. Lommatzsch 1940; Nilsson 1943; Basbous 1950; Schütt 1958), little progress has been made in this area. The evidence reviewed above suggests that it is entirely feasible not only to reduce but to completely eliminate lignin content of wood without unduly affecting other aspects of xylem development. However, complete elimination of lignin from the polysaccharide framework would support neither hydraulic conduction nor erect growth; thus, the minimum lignin level required for water conduction and structural support needs to be established. In view of the lower lignin content of hardwoods, a 10% decrease in conifers would appear to be feasible.

Lignin and wood specific gravity appear to vary independently. It is unlikely that specific gravity is more important than lignin as regards wood, pulp, or paper quality. Because of the complexity of wood specific gravity, there is no guarantee that increasing it will improve wood quality; decreased wood quality could also result. In contrast, alteration of lignin content in wood to an optimum level will certainly improve pulp yields and decrease pulping costs.

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DISEASE RESISTANCE FROM HYBRIDS OF
PINUS ECHINATA X *PINUS TAEDA*

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ABSTRACT

One of the "New Ways" which forest geneticists are using to improve disease resistance and wood production is interspecific hybridization. In the southern United States the hybrid of shortleaf pine x loblolly pine is proving to be highly resistant to the fusiform gall rust (*Cronartium quercuum* (Berk.) Miyabe ex Shirai f. sp. *fusiforme*), a major cause of mortality and growth loss in loblolly pine (*Pinus taeda* L.) and slash pine (*P. elliottii* Engelm. var. *elliottii*). Some F_1 hybrids from controlled pollination of selected parent trees, and most backcrosses of selected F_2 hybrids to selected loblolly pine have growth rates equal to, or better than, loblolly pine. Six years after planting in a seedling seed orchard, F_1's and backcrosses had, respectively, 44 and 34 percent less fusiform rust infection than loblolly pine. In a ten year old planting in a high hazard area in central Georgia 17 backcross hybrids averaged 51 percent less rust infection than pure loblolly pine, and were not significantly different from loblolly pine in wood volume production. Indications are that a fusiform rust resistant strain of loblolly pine can be achieved by interspecific breeding and selection, using shortleaf pine as the genetic source of disease resistance.

Keywords: Interspecific hybrids, *Cronartium quercuum* f. sp. *fusiforme*.

RÉSUMÉ

L'une des "Nouvelles manières" que les généticiens forestiers utilisent pour améliorer la résistance aux maladies et la production ligneuse est l'hybridation interspécifique. Dans le sud des États-Unis l'hybride entre le pin jaune et le pin à l'encens se révèle hautement résistant à la rouille-tumeur fusiforme (*Cronartium quercuum* (Berk.) Miyabe ex Shirai f. sp. *fusiforme*), cause majeure de mortalité et de perte d'accroissement chez le pin à l'encens (*P. taeda* L.) et le pin d'Elliott (*P. elliottii* Engelm. var. *elliottii*). Quelques hybrides F_1, issus de la pollinisation dirigée de parents sélectionnés et la plupart des rétro-croisements entre des hybrides F_2 sélectionnés et des pins à l'encens ont des taux de croissance égaux à ceux du pin à l'encens ou meilleurs. Six ans après avoir été plantés dans un verger à graines de semis, les F_1 et les rétro-croisements avaient, respectivement, 44 et 34 pour cent moins d'infection par la rouille fusiforme que les pins à l'encens. Dans une plantation âgée de 10 ans située dans une zone de haut risque dans le centre de la Géorgie, 17 hybrides rétro-croisés souffraient en moyenne de 51 pour cent moins d'infection par la rouille que les pins à l'encens de race pure, et n'étaient pas significativement différents de ces derniers pour ce qui est du volume ligneux produit. Des indices permettent de croire qu'une lignée de pin à l'encens résistante à la rouille fusiforme peut être développée par croisement interspécifique dirigé et la sélection, le pin jaune servant de source de gênes pour la résistance à la maladie.

Mots-clés: hybrides interspécifiques, Cronartium quercuum f. sp. fusiforme.

INTRODUCTION

One of the "New Ways" which forest geneticists are using to improve disease resistance and wood production is interspecific hybridization. In the southern United States the fusiform gall rust (Cronartium quercuum (Berk.) Miyabe ex Shirai f. sp. fusiforme) is a major cause of mortality and growth loss in loblolly pine (Pinus taeda L.) and slash pine (P. elliottii Engelm. var. elliottii). Shortleaf pine (P. echinata Mill.) is resistant to fusiform rust (Kraus and Powers 1984). Interspecific hybridization, backcrossing and selection are being used to develop a rust resistant strain of loblolly pine (Kraus and LaFarge 1978).

MATERIAL AND METHODS

In 1933, the artificial hybrid of shortleaf and loblolly pine was made at the Western Institute of Forest Genetics in Placerville, CA. Some of the resulting seedlings were established in a block planting at Placerville for measurement and observation. In 1949, a wind-pollinated crop of 55,000 seeds was collected from 60 of these trees. These seed constituted an F_2 generation and were distributed to interested research foresters in the south and southeastern United States.

In 1969, 18 years after outplanting, F_1, F_2, and backcross hybrids of shortleaf x loblolly pine obtained from Placerville and growing on the Hitchiti Experimental Forest near Macon, Georgia, were assessed for growth and disease infection (Sluder 1970). Fusiform rust infection levels of the hybrid trees were minimal compared to over 60% infection of loblolly pine. Volume production of one of the F_1 hybrids was not significantly different from loblolly pine. Two unreplicated blocks of F_2 hybrids were examined at age 20 and found to be totally free of fusiform rust infection. On the basis of these data and other reports (Henry and Bercaw 1956, Jewell and Henry 1959, Schmitt 1968), work began at Macon in 1972 to determine the feasibility of breeding a strain of loblolly pine resistant to fusiform rust based on the genetic resistance of shortleaf pine.

The parents of the F_1 made at Placerville had not undergone any sort of selection, so new F_1 crosses were made by controlled pollination of short-leaf pine and loblolly pine which had been selected for use in the seed orchards of the Georgia Forestry Commission. The loblolly pine parents used in the crosses were selected on the basis of the rapid growth of their progeny in previous field tests. The shortleaf pine used as parents were those which were flowering well in the seed orchard. Backcrosses were made by using the selected loblolly as pollen parents to cross with the selected F_2's in the block planting on the Hitchiti. Putative F_3's were obtained by collecting wind pollinated seed from these Hitchiti hybrid F_2's.

In 1974 a field planting of hybrid seedlings was made in a high rust hazard area in the upper coastal plain of central Georgia in Houston County. The planting contained three F_1's, all having the same shortleaf pine female parent; five F_3's; and 17 backcrosses. Three seedlots of shortleaf pine and two seedlots of loblolly pine were used as species checklots. Previous reports have detailed the results of the 3rd and 5th year assessments (LaFarge and Kraus 1977, 1980).

RESULTS AND DISCUSSION

The 10th year assessment was the first at which d.b.h. could be accurately measured and volumes calculated. In addition, average yearly height growth between 5 and 10 years was used as a parameter, along with survival and the percentage of trees which had never shown any symptoms of fusiform rust infection. The resulting data and the results of the statistical analysis are shown in Tables 1 and 2, respectively.

Table 1. Tenth-year results of a shortleaf x loblolly pine hybrid test in Georgia

Groups	Seedlots	Mean annual height growth	Tree volume	Trees rust free
	No.	m	m^3	Percent
(1) Shortleaf Pine	3	0.72 c	0.020 c	98 a
(2) F_1 Hybrids	3	.79 b	.030 b	98 a
(3) F_3 Hybrids	5	.76 b	.027 b	89 b
(4) F_2 Hybrids BC to Loblolly	17	.85 a	.038 a	58 c
(5) Loblolly Pine	2	.83 a	.040 a	7 d

Table 2. Analyses of differences among 10-year-old shortleaf x loblolly pine hybrids and their parent species

Source of Variation	D.F.	Mean Squares Height growth	 Volume	 Rust free
		x 10^3	x 10^5	Percent
Replications	3	37.56**	12.10	265.75
Seedlots	29	16.29**	23.91**	1965.34**
Within Group 1	2	19.56	5.45	222.30
Within Group 2	2	14.52	3.82	132.77
Wighin Group 3	4	2.85	2.55	545.95**
Within Group 4	16	7.42	9.41*	771.23
Within Group 5	1	.57	.54	37.88
Group 1 vs 2&3	1	30.34*	61.00**	706.84*
Group 2 vs 3	1	5.68	7.92	213.24
Groups 1,2,3 vs 4&5	1	235.31**	41.20**	29116.28**
Group 4 vs 5	1	2.22	4.26	231.73**
Rep. x Seedlot(error)	87	7.41	4.64	111.98

After 10 years in the field there were highly significant differences among the groups of seedlots related to the proportions of genetic material derived from the parental species. Stem volume and height growth of the shortleaf pine checklots were less than those of the F_1's and F_3's. Most of the variation in rust resistance resulted from the difference between the shortleaf pine, F_1's and F_3's combined vs the backcrosses and loblolly pine combined. Overall, the backcrosses averaged 58% rust free vs 7% rust free for the loblolly pine checklots; this difference was highly significant. Equally important was the fact that the backcrosses were not significantly different from loblolly pine in height growth rate and tree volume, indicating that a good level of fusiform rust resistance could be achieved with the interspecific hybrids without a loss of wood volume production. There was sufficient variation among the backcrosses in both rust resistance and volume production to indicate that by selective backcrossing substantial improvement in disease resistance can be achieved with no sacrifice in growth.

In 1978, a hybrid seedling seed orchard was established in cooperation with the Georgia Forestry Commission. This orchard contains hybrid progeny of 16 F_1's, 17 F_3's and 27 backcrosses. Initially this seed orchard contained 3,825 trees at a spacing of 1.5 x 2.4 meters, or approximately 1.4 hectares. The trees were planted in 17 replications, each containing 225 trees, with unequal numbers of trees per seedlot. One seedlot each of shortleaf pine and loblolly pine was included to provide estimates of the level of fusiform rust infection and comparisons of growth parameters.

After six years the shortleaf pine was significantly shorter than the hybrids and loblolly pine and had never been infected by fusiform rust (Table 3). Overall, the hybrid trees were not significantly shorter than the loblolly pine checklot, and they had sustained significantly less fusiform rust infection than the loblolly pine.

Table 3. Georgia Forestry Commission shortleaf x loblolly hybrid seedling seed orchard: sixth-year assessment

Group	Seedlots	Height	Trees free of rust
	No.	m	Percent
Shortleaf Pine	1	4.27 c	100.0 a
F_1 Hybrids	16	4.72 a	98.8 a
F_3 Hybrids	17	4.54 b	96.2 ab
F_2 Hybrids BC to Loblolly	27	4.84 a	89.2 b
Loblolly Pine	1	4.70 ab	54.7 c

This seedling seed orchard has now been rogued 3 times. At each roguing all trees having any symptoms of fusiform rust infection were removed. Additional trees were removed on the basis of growth rate and stem form when needed to improve the spacing. At this time approximately 1/3 of the original number of trees planted remain; of these approximately 30% have begun to flower and produce seed. This seedling seed orchard is now being put on a production status with the intention that the seed will be used to produce seedlings for planting in areas of known high fusiform rust hazard, and with the expectation that these seedlings will be approximately 40 to 50 percent more rust resistant than, and at least as fast growing as, loblolly pine.

The possibility exists that the hybrid variety of loblolly pine could serve as a genetic bridge for the development of a strain of fusiform rust which would be virulent on shortleaf pine. Years of natural hybridization have not produced such a strain of rust, and an artificial inoculation test which used inoculum collected from infected artificial hybrids (Kraus, Powers 1984) indicate that this may not be a problem.

We anticipate that there will be problems in the orchard with some trees which may not flower well, produce sterile pollen, or flower but fail to produce viable seed. By using fairly large populations, we expect that these problems can be surmounted by careful selection of trees going into the next generation. We also do not yet know what the result will be when we cross backcross trees x backcross trees, this will be occuring in the seedling seed orchard but we have no field tests yet which permit us to say whether we have achieved our goal of developing a strain of rust resistant loblolly pine by interspecific hybridization with shortleaf pine.

Until we can get these controlled cross tests in the field, we will be conducting wind pollinated progeny tests with seed collected from the backcross families in the seedling seed orchard.

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STEM QUALITY AND VOLUME OF DIFFERENT STRAINS OF WHITE SPRUCE FROM CANADA STUDIED IN MARYLAND

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ABSTRACT

White spruce (*Picea glauca* (Moench) Voss.), represented by nine geographic sources from Ontario and six from Québec, Canada, was studied in Garrett County, Maryland, at an elevation of about 850 m. At 22 years after planting all sources showed an outstanding survival (95%-100%), but varied in height from 91% to 107% of the plantation mean (8.6 m), in stem volume from 68% to 131% ($\bar{x}$ = 0.1 cu m), and the number of crooked or forked stems ranged from 2% to 22%. Heights and volumes were inversely correlated to the latitude of the seed source (r = -.70 and -.73, respectively). The most outstanding for growth rate were the sources from Carnarvon (45°04'N, 78°42'W) and South Monaghan (44°18'N, 78°18'W), Ontario. One of the best sources for stem form was from Québec and two were from Ontario. There was no significant relationship between the volumes of 22-year old trees and the heights of 5-year old trees. Heights and diameters in Maryland showed only a moderate relationship (r = .53 to .55) with the 15-year heights and volumes of the same sources in Minnesota.

RÉSUMÉ

L'épinette blanche (*Picea glauca* (Moench) Voss.), représentée par neuf provenances géographiques de l'Ontario et six du Québec, Canada, a été étudiée dans le comté de Garrett, Maryland, à une altitude d'environ 850 m. Vingt-deux ans après la plantation toutes les provenances indiquaient une survie remarquable (95-100%), mais variaient en hauteur entre 91 et 107% de la moyenne de la plantation (8.6 m), en volume de la tige de 68 à 131% ($\bar{x}$ = 0.1 m^3), et le nombre de tiges tordues ou fourchues variait de 2 à 22%. Les hauteurs et les volumes étaient inversement corrélés à la latitude de la source de graines (r = -.70 et -.73 respectivement). Les plus remarquables pour le taux de croissance étaient les provenances de Carnarvon (45°04'N, 78°42'W) et South Monaghan (48°18'N, 78°18'W) en Ontario. Une des meilleures sources pour ce qui est de la forme du tronc était du Québec et deux venaient de l'Ontario. Il n'y avait pas de relation significative entre les volumes des arbres de 22 ans et les hauteurs des arbres de 5 ans. Au Maryland, les hauteurs et les diamètres ne révélaient qu'un rapport modéré (r = .53 à .55) avec les hauteurs et volumes à 15 ans des mêmes provenances au Minnesota.

INTRODUCTION

White spruce (Picea glauca (Moench) Voss.) is one of the most important trees of Canada and the northeastern United States. It has a large natural range which stretches from New England and the Maritime provinces of Canada to Alaska, and is a valuable tree for pulpwood and many other uses. In Maryland, about 100,000 seedlings of P. glauca are planted annually, mainly in the Appalachian region.

Within its large range, this species is expected to be variable. Consequently, seed source research has been important and several provenance studies have been established. One such test was initiated in 1957 by the Institute of Forest Genetics at Rhinelander, Wisconsin; it included 24 seed sources from the entire range, and 14 research plantations scattered from New Brunswick to North Dakota. Another provenance test was started by Mark Holst of the Petawawa (Ontario) Forest Experiment Station. This study included seed sources from eastern Ontario and western Québec, with plantations established in Canada, Maryland, Minnesota and Michigan. Data reported from the plantations in Canada by Holst (1960, 1962), and Jones (1958), and from the experiments in the United States by Jeffers (1969), Nienstaedt and Teich (1972), Wilkinson et al. (1971) and Wright et al. (1977) have shown that white spruce exhibits a significant geographic variation. The results summarized by Nienstaedt (1969) indicated that its growth rate can differ at the ratio of one-to-four depending on the seed source.

Studies of different sources by Genys (1965) and Genys and Nienstaedt (1979) in Maryland helped in identifying some strains from southeastern Ontario which had outstanding heights at five or fewer years after planting. This present report form Maryland is based on measurements of trees at 22 years after planting, and the stem volume is used as the major criterion.

MATERIALS AND METHODS

This study is part of the provenance experiment with white spruce initiated by the Petawawa Experiment Station (renamed as the Petawawa National Forestry Institute) in Canada. Seeds were collected in Ontario and Québec, and in 1954-55, 2-0 seedlings were produced at Petawawa. In 1956, they were transplanted and grown for two years by the Lake States Forest Experiment Station in Wisconsin. A share of 2-2 seedlings, representing 17 different populations was shipped to Maryland's Department of Research and Education (now, the Center for Environmental and Estuarine Studies) in May, 1958, when it was too late for planting, and the seedlings were "heeled in" for one more year. The research plantation was established on April 16, 1959, when seedlings were five years old, and ranged in height from 15 to 27 cm.

The research plantation is located in the Collier Tract at Savage River State Forest, Garrett County, Maryland, which lies at 850 m above sea level and is a typical site of the Allegheny Plateau. Prior to planting, the site was pasture land. Seedlings were planted within 40 cm squares cleared of sod. Fifteen of the 17 sources of trees were planted in 20 randomized blocks. Each block included each source, represented by a two-seedling plot; trees were spaced at 2.4 m within rows and 3.0 m between rows.

Heights of young trees were measured at five years after planting (Genys, 1965). The present study was conducted in the fall of 1980, when the specimens were 27 years old from seed, grown 22 years after planting. Heights were measured with a pole, and diameters at 15 cm above the ground were measured with a tape. The stem volume of each tree was calculated by the equation: V = H x B x 0.333, where "H" is total height, "B" is basal area at 15 cm above the ground, and 0.333 is a uniform form factor. Also, counts were made of dead trees, and of trees with crooks and forks. The analysis of variance was based on plot means. It included a total of 299 degrees of freedom (DF): 19 DF for blocks, 14 DF for provenances and 266 DF for error. Duncan's multiple range test was used to determine the least significant differences (LSD), and the largest LSD (for p = 14) was used to identify the differences. Correlation coefficients were calculated to evaluate the relationships among the studied characteristics, and their relationship to geographic factors and to data from Minnesota.

RESULTS

Survival

Because of sturdy planting stock and favorable site conditions, survival was outstanding. Only 6 of the 600 trees planted died. Survival rates of trees from diverse seed sources varied from 95% to 100%. These differences, however, were statistically (at 0.05 level) non-significant.

Stem quality

Stem quality of trees was judged on the basis of number of crooked or forked stems. Overall, 12% of the trees had these defects. Most of the crooks developed at an early age (on the lower parts of the stem). Among individual populations the proportions of defective stems ranged from 2.5% to 22.5%. The fewest trees with crooks or forks (only 2.5%) were among the sources from St. Maurice River, Québec (#61), Holland Township (#70) and Maple Leaf (#47); last two from Ontario. In contrast, the population from Maniwaki (#60) included more than 22% of the trees with defects. Also, 15% or more trees were crooked in four other populations (Table 1). The percentages of trees with defects showed no significant relationship to either the growth rate or the geographic factors such as longitude, latitude or elevation of the seed source (Table 2).

Height

Twenty-two years after planting the trees had an average height of 8.58 m, and the heights of different geographic strains ranged from 91% (7.8 m) to 109% (9.4 m) of the experiment mean (M). Among the nine populations from Ontario, four were taller than the plantation mean. The two most outstanding sources from Carnarvon (#49) and So. Monaghan (#66), grew 7% to 9% taller than the plantation mean, and 16% to 18% faster than the poorest strain from Potter, Ontario (#54). Among the sources from Québec, the tallest trees (103% of M) were from L'Annonciation (#56), and the smallest (96% of M) from

Trois Pistoles (#71). Height variation among the Québec sources, however, was not significant (at 0.05 level).

The trees from the northern sources grew slower than those from more southern sources, and at all ages (5-, 10-, and 27 years after germination) the heights were inversely correlated to the northern latitude of the seed source (r = -.20, r = -.64, and r = -.70, respectively). Apparently, this relationship became stronger as trees became older and grew taller. There was no significant correlation between the height and either the longitude or the altitude of the seed source.

Table 1. Growth rates and other characteristics of white spruce (Picea glauca) from 15 different sources in Canada studied 22 years at the Savage River State Forest in Garrett County, Maryland

Data Code:		(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
Seed	Origin of seed source in Canada				Trees	Tree Height		Tree Volume	
lot no.	Province, Location	Latitude N	Longitude W	Altitude	with forks	Real	Rel. to mean	Real	Rel. to mean
				m	%	m	%	cu m	%
49	Ontario, Carnarvon	45°04'	78°42'	320	7.5	9.16	107	.126	131
66	Ontario, So. Monaghan Twp.	44°18'	78°18'	244	17.5	9.36	109	.119	123
45	Ontario, Vankleek Hill	45°30'	74°40'	76	7.5	8.31	97	.107	111
70	Ontario, Holland Township	44°25'	80°50'	366	2.5	8.41	98	.106	109
46	Ontario, Denbeigh	45°05'	77°20'	335	7.5	8.93	104	.103	106
47	Ontario, Maple Leaf	45°15'	77°50'	381	2.5	8.76	102	.101	104
68	Ontario, Essa Township	44°18'	79°50'	290	5.1	8.56	100	.096	99
61	Quebec, St. Maurice River	46°50'	72°46'	152	2.5	8.49	99	.094	97
60	Quebec, St. Charles De Mond.	46°21'	73°21'	152	18.4	8.45	98	.094	96
71	Quebec, Trois Pistoles	48°08'	69°10'	122	5.1	8.25	96	.093	96
56	Quebec, L'Annonciation	46°25'	74°52'	274	10.2	8.81	103	.091	94
55	Quebec, Maniwaki	46°25'	75°52'	274	22.5	8.53	99	.088	91
59	Quebec, St. Zenon	46°35'	73°50'	457	15.0	8.32	97	.085	88
50	Ontario, Sand Lake	45°38'	79°10'	396	10.0	8.52	99	.084	86
54	Ontario, Potter	48°52'	80°51'	274	15.0	7.80	91	.066	68
The least significant difference at 0.05 level (p = 14)					21.0	0.83	9.7	.031	32
The F-value.					1.64	4.02	4.02	3.94	3.9

(6) and (8): Mean height was 8.58 m; mean volume - 0.097 cu m

The height of trees at 22 years after planting showed a moderate but statistically significant correlation with the height at five years after planting (r = .55), but no significant correlation to the height of trees at the time of planting (r = .31). Also, the rank of heights of different populations changed. For instance, source No. 46 from Denbeigh, Ontario, which ranked first at five years after planting, ranked only third at 22 years.

Table 2. Relationship among the studied characteristics of different geographic strains of white spruce, and their relationship to the latitude, longitude and altitude of the provenance. Data codes are the same as in Table 1; AP = after planting. Included are some correlation coefficients based on data (M, N) from Minnesota (Stellrecht *et al.*, 1974).

Data code:	(1)	(2)	(3)		(A)	(B)	(5)	(7)
Y_2:	Provenance				Heights at years (AP)			Volume
Y_1:	Latitude	Longitude	Altitude	Forked trees	0	5	22	22 yrs MD
(4) Forked trees (22 yrs. AP)	.22	-.04	.00	1.00				
(A) Height (AP)	-.20	.17	.17	-.02	1.00			
(A) Height (5 yrs. AP)	-.64**	.22	.29	-.24	.63**	1.00		
(5) Height (22 yrs. AP)	-.70**	.16	.18	-.02	.32	.55*	1.00	
(7) Volume (22 yrs. AP)	-.73**	.09	-.10	-.27	.27	.36	.79**	1.00
(M) Height (15 yrs. in MN)	-.45*	--	.27	-.23	.66**	.61**	.55**	.40
(N) Volume (15 yrs. in MN)	-.59**	--	.18	-.36	.71**	.71**	.53*	.53*

* significant at 0.05 level
** significant at 0.01 level

Stem volume

The mean volumes of trees from different seed sources ranged from 0.066 cu m to 126 cu m, or from 68% to 131% of the experimental mean (0.098 cu m). The most outstanding source for volume growth came from Carnarvon, Ontario (#49). Its volume was about twice as large as that of the strain from Potter, Ontario (#54). The second best population came from South Monaghan Township in Ontario (No. 66); its stem volume was 23% larger than the experimental average.

Volumes of trees from different geographic sources showed a strong inverse correlation with the northern latitude ($r = -.73$), but no significant relationship to either the longitude or the altitude of the seed source. In broad geographic terms, the sources with superior volumes were from the region north of Lake Erie and east of Lake Huron. The best source from the Province of Québec was #61, from St. Maurice River.

It would be beneficial to obtain volume estimates by measuring heights alone. However, when dealing with different strains, the estimates may be misleading. Some trees can be tall as a result of competition for upper space, but their diameters can be small. In this experiment, 22-year volumes and 22-year heights showed a significant correlation ($r = .79$), but the determination coefficient was only $r^2 = .62$. The height of trees at five years after planting showed no significant relationship with the 22-year volumes ($r = .36$, $r^2 = .13$) and may have been a poor indicator of the future tree volume.

DISCUSSION

The results of this experiment in Maryland indicated that all 15 strains of white spruce from the southern sections of Ontario and Québec, Canada, exhibited outstanding survival. Their heights and stem volumes, however, were highly variable. In comparison to the poorest strain, the best strain had a larger height by about 20%, and a larger volume by 90%. Apparently, the best strain may produce nearly twice as much wood as the poorest. Selection of the most productive strains (for volume) based on heights of young trees may have been inefficient. For instance, at five years after planting, the population No. 46 from Denbeigh, Ontario had the largest heights (Genys 1965). However, at 22 years after planting it ranked only third best in height and only fifth best in volume. At 22 years, the strain with the largest volume was No. 49 from Carnarvon, Ontario. Also, this population had a relatively good stem quality.

In general, the farther north the seed source, the slower was the growth rate of the respective trees. This trend became more evident as the trees grew older. Heights and volumes of trees in Maryland showed a moderate relationship (r = .53 and r = .55 respectively) with these traits of the same populations studied for 15 years in Minnesota (Stellrecht et al., 1974). The Carnarvon source which ranked No. 1 in volume production in Maryland, ranked No. 2 in Minnesota.

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PROBLEMS OF "IN VITRO" MICROPROPAGATION OF BLACK LOCUST (ROBINIA PSEUDOACACIA L.)

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ABSTRACT

Exclusively an introduced species in Romania, black locust is today widespread all over the country. As a forest tree it is remarkable for its high wood productivity, rusticity and its use for beekeeping. That is why a breeding program is under way. In the framework of this program, tissue culture, first of all as a means of propagation of genetically improved reproductive materials obtained by "conventional" breeding methods, is the main point of interest.

Tissue culture research for the purpose of micro-propagation of black locust started only recently. In the present paper we discuss the problems of "in vitro" micro-propagation, mainly the correct ratio between shoot formation and root formation, as well as the economic feasibility of producing more plantlets per square meter of laboratory space.

The next step will be about continued progress in developing practical systems for mass propagation, mostly hybrids.

Keywords: Sterilization, organogenesis, multiplication rate.

RÉSUMÉ

En Roumanie le robinier faux-acacia est une espèce introduite mais aujourd'hui, il est répandu partout dans le pays. Comme arbre forestier il est remarquable par sa haute production en bois, par sa rusticité et par son utilité en apiculture. Ce sont les raisons qui ont déterminé l'établissement d'un programme d'amélioration de cette espèce. Dans ce programme, la culture des tissus, comme outil pour la multiplication du matériel de reproduction amélioré obtenu par des méthodes conventionnelles, est de premier intérêt.

Les recherches concernant la culture des tissus pour la micropropagation du robinier faux-acacia ont débuté récemment. Dans le texte présenté ici sont discutés les problèmes posés par la micropropagation "in vitro", principalement le rapport entre la formation de la tige et la formation de la racine, ainsi que la faisabilité économique de la production de plus de plantules par mètre carré de surface de laboratoire.

Le point suivant sera le progrès continu dans le développement des systèmes pratiques de propagation en masse, spécialement des hybrides.

Mots-clés: stérilisation, organogénèse, taux de multiplication.

INTRODUCTION

Black locust (Robinia pseudoacacia L.), a species introduced in Romania a long time ago, is at present widely spread, both in forest culture and in settlements, or along the roads. As a forest tree it is remarkable for its high wood yield and short production cycles, for its esthetic value and for its contribution to honey production. Therefore, a genetic improvement program was initiated for black locust trees. Besides the "conventional" methods for improvement and for multiplication of reproductive materials, "in vitro" micropropagation is also envisaged. To this end, research has recently been started, aiming at establishing suitable biotechnologies for black locust tissue culture.

The specialized literature signals out certain results of black locust tissue culture. Trippi (1963) surveyed callus proliferation with explants made up of internodes from young and adult branches and he could not detect any seasonal effects with explants taken from the young parts of the tree; explants from adult zones proliferated less if taken while flowering. General references, but no results of specific research, are to be found in the papers published by Sommer and Brown (1979) and Brown (1980). Brown (1981), making reference to locust in a survey on tissue culture application to woody biomass production, quotes an unpublished paper by Sommer (1978) concerning explant origin; as for the culture medium, for dicotyledonous plants as a whole, he only suggests a modification of the one used by Blaydes. Data on multiplication rate and duration as well as costs as against classical methods are interesting since they emphasize wide and genuine prospects for the use of "in vitro" micropropagation.

Also, mention should be made of a paper by Brown and Sommer (1982) where locust is frequently referred to, together with other angiosperm.

Finally, mention should be also made of a paper by Fukuka and Sakurai (1982) dealing with lignin growth and storing in cell culture.

Our research set out from this respective level of knowledge and aimed at establishing a biotechnology for the micropropagation of improved reproductive material for locust. The present paper approaches certain main aspects related to black locust tissue culture.

MATERIAL AND METHOD

Seeds decorticated before and after sterilization and before scarification were used as initial culture material.

As for the seeds previously decorticated, sterilization was achieved according to the following method (S1): 25 minutes stirring in 10% calcium hypochlorite solution, washed twice, for 10 minutes each time, in sterile distilled water, 15 minutes in sterile distilled water, UV exposed and afterwards, inoculated.

The post-sterilization decorticated or scarified seeds were washed for 3 hours under tap water flow, were stirred for 15 minutes in mercuric chloride solution 0.1 plus 3 drops of Tween 80, were washed twice -- 10 minutes each time -- in sterile distilled water (S2) and were then inoculated. The same material was also sterilized by another method: 1 minute stirring in ethylic alcohol, 2 times stirring in a sterile solution of calcium chloride (10 mg/l) 10 minutes each, 2 times washing in sterile distilled water (S3) 10 minutes each.

Inoculation was performed on White and Risser (1964) culture medium and on filter paper with sterile tap water. Inoculation was carried out in test-tubes (Ø=25 mm) with 12 ml culture medium.

Plantlets were obtained from explants which had been taken from 1-2 cm long shoot tips, 0.5-1 cm long stem segments without auxiliary buds and 0.5-1 cm long radicel tips. Such explants were inoculated on White and Risser (1984) basic medium without 2.4-D, with and without 20 mg/l activated charcoal 5.5 pH and Difco agar 7 g/l.

After differentiation, four weeks later, the plantlets were transferred on Murashige and Skoog (1962) medium with the following adaptations:

(1) BAP 1 mg/l with 0.1% activated charcoal
(2) BAP 1 mg/l without activated charcoal
(3) BAP 2 mg/l with 0.1% activated charcoal
(4) BAP 2 mg/l without activated charcoal

With all variants the pH was 5.7 and the agar was 7 g/l.

Four to six weeks later, plantlets were transfered to Murashige and Skoog (1962) medium with two adaptations: (1) IAA 1 mg/l and (2) IAA 2 mg/l (5.7 pH, 7 g/l agar).

With a view to increasing the multiplication efficiency, an attempt was also made for prolongation on Murashige and Skoog (1962) with 1 mg/l GA, with and without 20 mg/l activated charcoal.

Initiation, differentiation, elongation, etc. cultures were set in 100 ml Erlenmeyer recipients with 20 ml medium. The culture medium was autoclaved at 112°C for 30 minutes.

In the growing room temperature was 23-25°C, while the photoperiod was 16 hours light and 8 hours dark. The growing room was air-conditioned. The transfers were performed in sterile rooms in an horizontal laminar flow previously desinfected by a Grolux Sylvania bactericide lamp.

RESULTS AND DISCUSSION

After the first survey it seems that seeds decorticated after sterilization by the S2 and S3 methods and germinated on filter paper in sterile water offer best results. But if we take into consideration their reactivity in the stages following micropropagation we can note that it was more reduced, that is the germination medium influences the subsequent stages -- as will be further evident -- to such an extent that it cannot be overlooked.

Considered from such an angle, seeds decorticated after sterilization by the S2 method and germinated on White and Riesser medium all germinate, with a relatively low infection rate. V6 registers almost similar values. Seeds decorticated before sterilization by the S1 method do not germinate satisfactorily, while the ones decorticated after sterilization by the S3 method register too high a rate of infection.

The explant origin plays a major role in the differentiation process. Using the White and Riesser medium, both with and without activated charcoal, the number of viable plantlets was relatively high, being higher with activated charcoal (75% as against only 66% without activated charcoal). The stem segments, although recommended by Brown (1980) for classical cutting, offered poor results both with and without activated charcoal (8%). Root tips were not reactive on the medium used.

In the differentiation stage on the Murashige & Skoog medium the 0.1% activated charcoal made all the plantlets register active growth, without axillary buds and in certain cases, with radicles forming when 1 mg/l or 2 mg/l BAP was used. When activated charcoal was not used, plantlets survived but only a few of them prolonged (20-25%).

The multiplication transfer onto Murashige & Skoog medium with 2 mg/l IAA resulted in 100% viable plantlets, very prolonged, with rich axillary light-green offsprings. Cutting down IAA to only 1 mg/l brought down the number of plantlets with elongation and ramification to 50% of the initial number. As an average, from one plantlet on Murashige & Skoog medium with 2 mg/l IAA 9 explants were obtained, with a range of 6-15 explants. That is a 1:9 reproductive rate in 6-8 weeks. Brown and Sommer (1982) mention with Populus, Acer and Robinia a 1:5 multiplication rate in 12 weeks, which is quite close to the results of our research, taking into account that the higher multiplication rate is finally reduced by the longer multiplication time.

Adding 1 mg/l gibberellic acid to the Murashige & Skoog medium did not bring about an additional elongation; the explants stopped growing, withered and died.

One of the essential aspects of black locust tree micropropagation is to achieve radicle formation without impeding shoot formation and elongation, that is not to develop roots before obtaining an adequately high multiplication rate, for the operation to be efficient. Roots appeared in the presence of BAP and activated charcoal or of IAA 2 mg/l. Another working hypothesis is that the roots could play, to a certain extent, a positive role in the formation and elongation of axillary shoots. This hypothesis is under investigation and certain results reported by Sommer (1978) support it. A particular sequence of the stages of micro-propagation has also proved important, as early as the germination stage. Similarly, direct multiplication on Murashige & Skoog medium with 2 mg/l IAA using explants from germinated seeds did not produce the same results -- 100% -- as in the case when all the above mentioned micropropagation stages were observed.

The research under way also considers the possibility of using roots as explants, as black locust roots very well naturally, in addition to the "in vitro" transfer conditions.

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V3 - Seeds decorticated after sterilization by the S3 method and germination on White and Riesser medium.

V4 - Seeds decorticated after sterilization by the S2 method and germination on filter paper.

V5 - Seeds decorticated after sterilization by the S3 method and germination on filter paper.

V6 - Seeds scarificated after sterilization by the S2 method on White and Riesser medium.

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